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STUDIES OF CARBOXYL-C^{14}-LABELED 3-INDOLEACETIC ACID IN PLANTS[1,2,3]

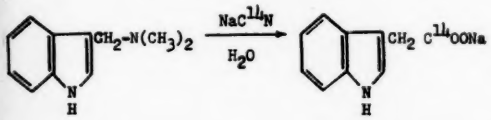
S. C. FANG AND JOSEPH S. BUTTS

DEPARTMENT OF AGRICULTURAL CHEMISTRY, OREGON AGRICULTURAL EXPERIMENT STATION, OREGON STATE COLLEGE, CORVALLIS, OREGON

Studies on the absorption, transport and metabolism of synthetic growth regulators using radioactive tracers have been carried out by many workers. In contrast, little work has been reported regarding the natural auxin, indoleacetic acid, which is of great interest to plant physiologists. This paper reports the results of experiments on the absorption and translocation of IAA-1-C^{14} in bean plants, and also the effects induced by 2,4-D treatment and light on the destruction of IAA in vivo.

MATERIALS AND METHODS

The synthesis of IAA-1-C^{14} was carried out according to the following scheme:



Gramine Indoleacetate

This method is essentially that of Stutz et al (10). The following procedure gave the highest yield based on $NaC^{14}N$. In a 25-ml Pyrex test tube, 350 mg (2 mM) of gramine (purchased from Nutritional Biochemicals Corporation), 49 mg of $NaC^{14}N$ (1 mM) and 40 mg of NaOH (1 mM) were dissolved in 10 ml of 50 % methanol solution. After cooling the solution in a dry ice bath, the tube was momentarily evacuated, flushed with nitrogen gas, and sealed. The mixture was then autoclaved at 20 lb pressure for 19 hours. At the end of the reaction, the seal was broken and the contents were transferred to a flask. The solution was first evacuated to remove the methanol and was then extracted four times with five-ml portions of ether to remove the excess gramine. After chilling to 0° C and adjusting the pH to 3.0 the IAA precipitated directly from the aqueous solution. The precipitate was filtered immediately and washed with a small amount of ice water. A yield of 105 mg with an activity of 6.5×10^7 cpm/mM was obtained, M.P. 166°–168° C after recrystallization. The sample was also chromatographed using isopropanol–water–ammonium hydroxide (10 : 1 : 1) as the developing solvent. The position of radioactive spot coincided with that of pure IAA.

Bean plants (*Phaseolus vulgaris* L., var. Black Valentine), corn plants (*Zea Mays* L., var. Golden Cross) and pea plants (*Pisum sativum* L., var. Alaska) were grown under greenhouse conditions in flats containing Chehalis sandy loam soil. A 95 % ethanol solution containing 0.1 % IAA-1-C^{14} and 0.2 % Tween-20 was used for treatments. The bean plants were treated when the primary leaves were almost fully expanded and the terminal buds were still very small. The solution was applied quantitatively to each plant along the midrib of the primary leaf by means of a microsyringe. After harvesting, all plants were sectioned into leaf, stem and roots; the sections were pooled and homogenized with 80 % ethanol. The corn plants were treated when they had grown to the fourth leaf stage. The pea plants were used 10 to 14 days after germination.

Forty uniform bean plants were selected and treated with 43 μgm IAA-1-C^{14} per plant in the midrib of one primary leaf. Ten plants were harvested 1, 3, 7, and 14 days after treatment. Eight plants from each group were weighed, sectioned, composited and homogenized with 80 % ethanol while the remaining two were used for making radioautograms. One ml aliquot of the ethanol solution from each composite was dried in cupped planchets and the C^{14} activity was measured with a thin mica window G-M counter (1.9 mg/cm^2).

Since the increase in rate of oxidation of IAA in vitro by light has been demonstrated (5), an experiment was designed to study the effect of light on the destruction of IAA in vivo. Eight uniform bean plants were treated with IAA-1-C^{14} (50 μgm per plant) on both primary leaves. The plants were placed in bell jars, four plants to each jar. One jar was covered with light-proof black paper, while the other was exposed to fluorescent light (1200 ft-c intensity) continuously except for the period from 10 P.M. and 6 A.M. The respiratory CO_2 was swept out continuously from the system, either with CO_2-free air or with O_2 and collected in NaOH solutions.

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The trapping solutions were replaced periodically with fresh NaOH. The carbonate in the solutions was precipitated as $BaCO_3$ and the radioactivity was measured in the usual manner. This type of experiment was carried out twice with bean plants, once with corn plants, and once with pea seedlings. In each case eight plants were used.

The enhancement of IAA oxidation in vitro by 2,4-D has been reported previously (4, 6). Recently, it was shown that the apparent enhancement of oxidation of IAA by 2,4-D was caused by an impurity (2,4-dichlorophenol). With pure 2,4-D no effect of 2,4-D on the oxidation of IAA in vitro has been reported (7). This experiment was carried out as an in vivo study to determine whether or not the 2,4-D exerts its action on the enzyme systems controlling the destruction of IAA in plants. Several experiments were performed. In the first run, 80 uniform bean plants were divided into four groups of 20 plants each. Each plant was treated with 21.8 μgm of IAA-1-C^{14} on one of the primary leaves. After completion of IAA treatment, three groups of plants were again treated on the same leaf with either 5, 10, or 20 μgm of non-radioactive 2,4-D (recrystallized twice from benzene and showing no effect on the oxidation of IAA in vitro), respectively, while the 4th group received no 2,4-D treatment. Four plants from each group were harvested at various intervals, and were divided into leaf, stem and roots samples on which radioactivities were determined. A second experiment was carried out with 16 plants in each group. The IAA-1-C^{14} was applied at the same level. Each plant in the four groups received 0, 1, 5, and 50 μgm of 2,4-D, respectively. In order to demonstrate further the effect of 2,4-D an experiment with a direct measurement of radioactive respiratory CO_2 from IAA-1-C^{14} treated bean plants was carried out. Each of eight uniform bean plants received 50 μgm of IAA-1-C^{14}. Four of these plants received an additional 50 μgm of non-radioactive 2,4-D per plant. Immediately after the application of chemicals, these two groups of plants were put separately into two bell jars and the amounts of radioactive CO_2 given off were collected and measured periodically as described

FIG. 1. The accumulation pattern of C^{14} in the bean plant. The primary leaf, on the right, received 43 μgm of indoleacetic acid-1-C^{14} the previous day.

TABLE I

ABSORPTION AND TRANSLOCATION OF C^{14} IN BEAN PLANTS WHICH RECEIVED 43 μGM OF 3-INDOLEACETIC ACID-1-C^{14} TREATMENT PER PLANT

	1 DAY		3 DAYS		7 DAYS		14 DAYS	
	TOTAL ACTIVITY	RECOVERY	TOTAL ACTIVITY	RECOVERY	TOTAL ACTIVITY	RECOVERY	TOTAL ACTIVITY	RECOVERY
	cpm	*%*	*cpm*	*%*	*cpm*	*%*	*cpm*	*%*
Treated leaves	52250	87.5	48750	81.3	39300	65.5	33850	56.4
Petioles, treated leaves	6700	11.2	4450	7.4	4000	6.7	5050	8.4
Untreated leaves	50	0.1	50	0.1	0	0	0	0
Petioles, untreated leaves	0	0	0	0	0	0	0	0
Terminal buds	200	0.3	700	1.2	1050	1.8	1100	1.8
First internodes	700	1.2	900	1.5	600	1.0	700	1.2
Hypocotyls	450	0.8	650	1.1	400	0.7	600	1.0
Roots	250	0.4	400	0.7	250	0.4	500	0.8
Totals		101.5		93.3		76.1		69.6

Plants were harvested 1, 3, 7, and 14 days after treatment. Total activity applied to each group of 8 plants = 60,000 cpm. All results were counted by a thin mica window G-M counter (1.9 mg/cm^2).

previously. The same type of experiment was repeated once, using corn as test plants.

Results and Discussion

The results of absorption and translocation of IAA-1-C^{14} in bean plants are shown in table I. Excluding the activity from the blade of the treated leaves, between 10 to 14 % of the C^{14} activity was absorbed and transported in all four groups. The amount of total recovery of C^{14} decreased with time, indicating that part of the applied IAA had been metabolized, the radioactivity appearing in the respiratory CO_2. The concentration of C^{14} in the petiole of the treated leaf, first internode and hypocotyl, reached the maximum in the first day. The accumulation of C^{14} in the terminal bud continued and it

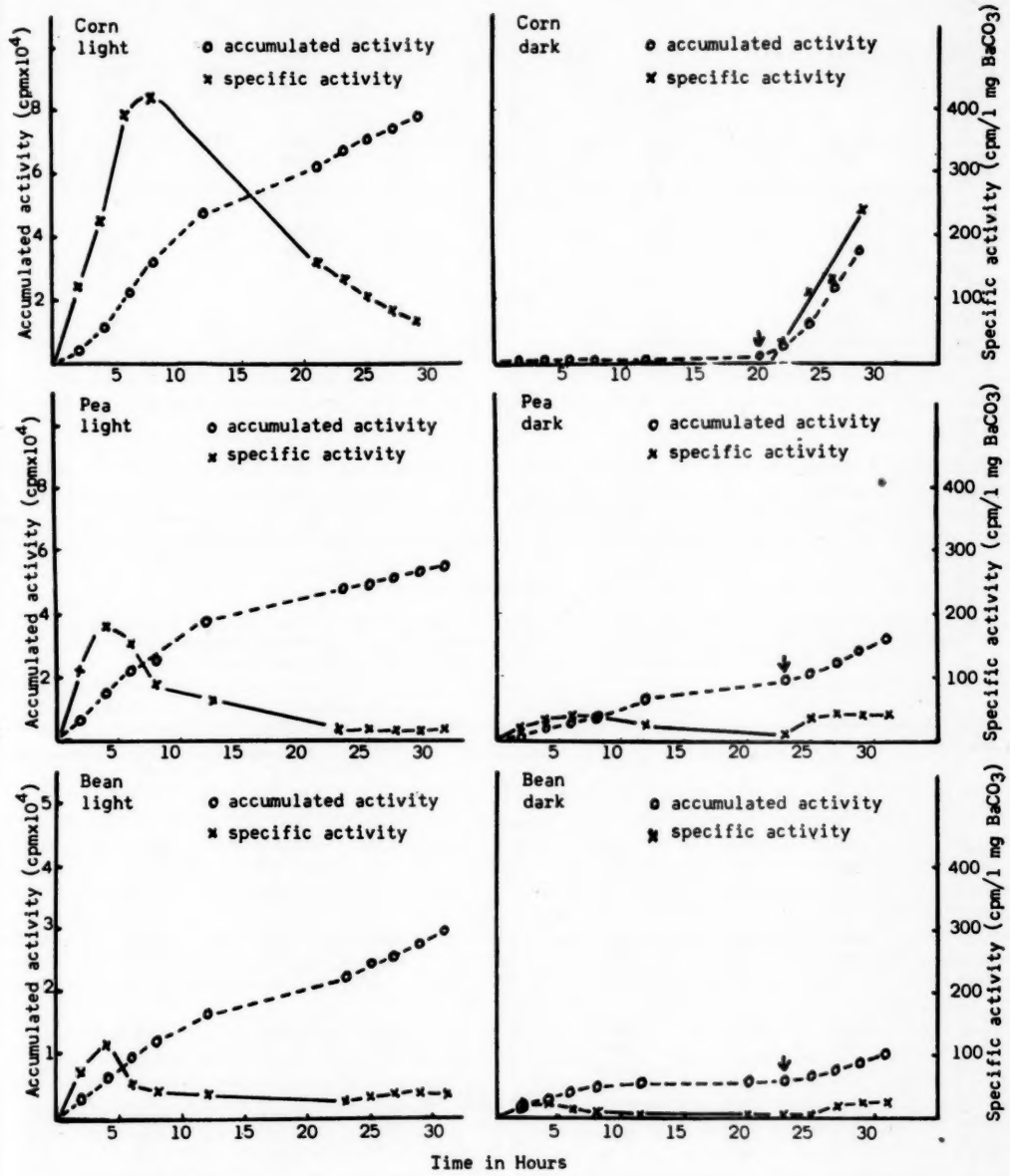

Fig. 2. Effect of light on the accumulative and specific activity of respiratory CO_2 in plants which received indoleacetic acid-1-C^{14} treatment. At arrow, black paper was removed and the plants were exposed to light.

TABLE II

EFFECT OF 2,4-D ON THE ABSORPTION AND TRANSLOCATION OF C^{14} IN BEAN PLANTS WHICH RECEIVED 21.8 μGM OF 3-INDOLEACETIC ACID-1-C^{14} TREATMENT PER PLANT. (IAA AND 2,4-D WERE APPLIED SIMULTANEOUSLY ON ONE OF THE PRIMARY LEAVES)

PLANT PART	3 DAYS		4 DAYS		7 DAYS		11 DAYS		17 DAYS	
	TOTAL ACTIVITY, CPM	RECOVERY, %	TOTAL ACTIVITY, CPM	RECOVERY, %	TOTAL ACTIVITY, CPM	RECOVERY, %	TOTAL ACTIVITY, CPM	RECOVERY, %	TOTAL ACTIVITY, CPM	RECOVERY, %
					Control					
Leaf	3600	24.6	3250	22.2	2300	15.8	1000	6.9	550	3.8
Stem	550	3.8	750	5.1	700	4.8	650	4.5	750	5.1
Root	0	0	0	0	0	0	0	0	0	0
					5 μgm 2,4-D					
Leaf	5100	34.9	5300	36.3	4200	28.8	3200	21.9	2600	17.8
Stem	300	2.1	100	0.7	300	2.1	300	2.1	150	1.0
Root	0	0	0	0	0	0	0	0	0	0
					10 μgm 2,4-D					
Leaf	6550	44.9	6200	42.5	4550	31.2	2950	20.2	3100	21.2
Stem	500	3.4	300	2.1	100	0.7	400	2.7	250	1.7
Root	0	0	0	0	0	0	0	0	0	0
					20 μgm 2,4-D					
Leaf	5450	37.3	4050	27.7	4400	30.1	3500	24.0	2800	19.2
Stem	150	1.0	600	4.1	350	2.4	300	2.1	200	1.4
Root	0	0	0	0	0	0	0	0	0	0

Total activity applied to each group of 4 plants = 14,600 cpm. A thin mica window G-M counter (1.9 mg/cm^2) was used.

did not reach a maximum until the 7th day after treatment. No activity or only a trace of C^{14} was noted in the untreated primary leaves. The accumulation pattern of C^{14} in bean plants after having been treated for 1 day with IAA-1-C^{14} is shown in figure 1. When these results are compared with a previous study of the rate of translocation of C^{14} in bean plant receiving labeled 2,4-D (2) it is clear that

TABLE III

EFFECT OF 2,4-D ON THE ABSORPTION AND TRANSLOCATION OF C^{14} IN BEAN PLANTS WHICH RECEIVED 21.8 μGM OF 3-INDOLEACETIC ACID-1-C^{14} TREATMENT PER PLANT. (IAA AND 2,4-D WERE APPLIED SIMULTANEOUSLY ON THE SAME PRIMARY LEAF)

PLANT PART	7 DAYS		11 DAYS		14 DAYS		18 DAYS	
	TOTAL ACTIVITY, CPM	RECOVERY, %	TOTAL ACTIVITY, CPM	RECOVERY, %	TOTAL ACTIVITY, CPM	RECOVERY, %	TOTAL ACTIVITY, CPM	RECOVERY, %
				Control				
Leaf	3700	25.0	2250	15.2	2850	19.3	2050	13.8
Stem	1200	8.1	1400	9.5	500	3.4	1050	7.1
Root	0	0	0	0	0	0	0	0
				1 μgm 2,4-D				
Leaf	4100	27.7	2600	17.6	2850	19.3	2300	15.5
Stem	950	6.4	1400	9.5	1350	9.1	500	3.4
Root	0	0	0	0	0	0	0	0
				5 μgm 2,4-D				
Leaf	4400	29.7	3900	26.4	3350	22.6	2300	15.5
Stem	1400	9.5	950	6.4	1050	7.1	1450	9.8
Root	0	0	0	0	0	0	0	0
				50 μgm 2,4-D				
Leaf	7200	48.6	5700	38.5	5200	35.1	5000	33.8
Stem	1450	9.8	2000	13.5	1700	8.7	950	6.4
Root	0	0	0	0	0	0	0	0

Total activity applied to each group of 4 plants = 14,800 cpm. A thin mica window G-M counter (1.9 mg/cm^2) was used.

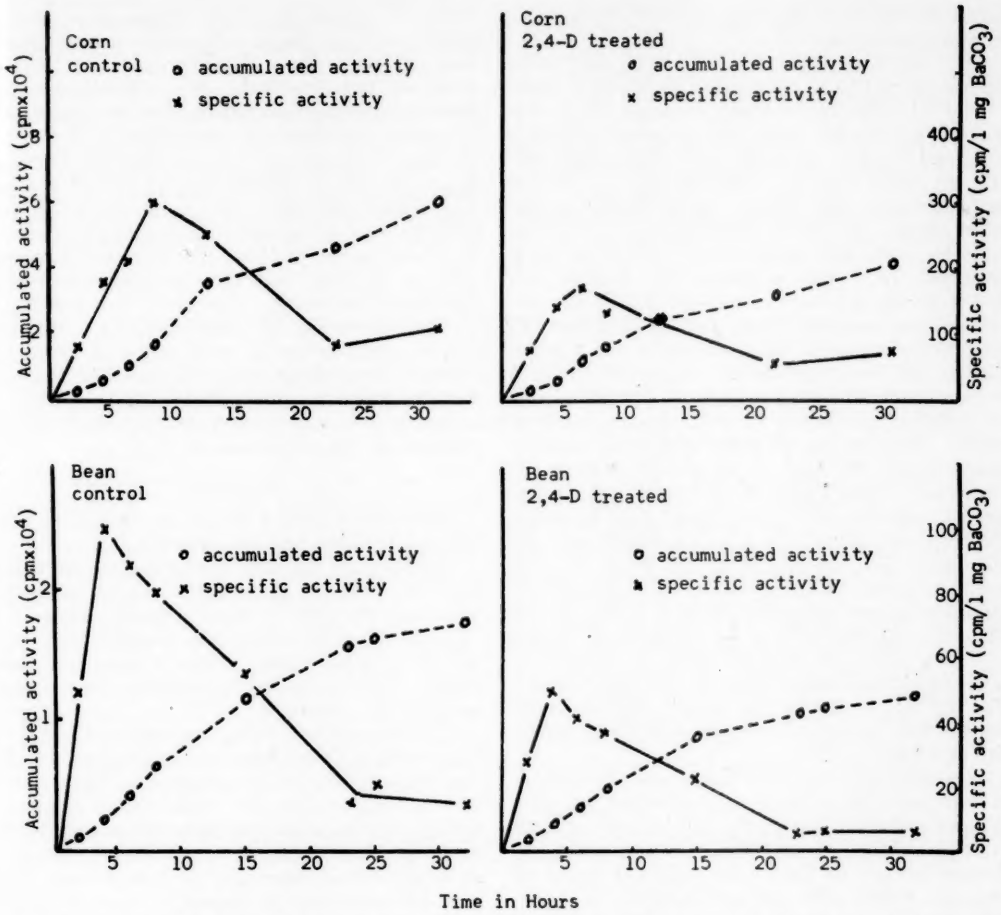

FIG. 3. Effect of 2,4-D on the accumulative and specific activity of respiratory CO_2 in plants which received indoleacetic acid-1-C^{14} treatment.

the C^{14} of the IAA does not translocate as readily. A larger portion of it remained in the treated leaf and may be gradually metabolized. Chromatographic study of 80 % alcoholic extract of IAA-1-C^{14} treated bean leaves revealed the presence of six compounds containing carbon-14. The R_F of these compounds agree quite well with the values reported by Andreae and Good (1) and Good et al (8). Detailed study of these IAA metabolites will be reported in a following paper.

The effect of light on the destruction of IAA in plants is represented in figure 2. It is clearly shown that the plants which were kept in darkness did not produce radioactive CO_2 from IAA-1-C^{14} at the same rate as the plants expose to light. As soon as the plants in the dark were exposed to light, the rate of radioactive CO_2 evolved was greatly accelerated. The destruction of IAA in corn plants depends completely on light, as negligible amounts of $C^{14}O_2$ were given off by the plants during the dark period. On the contrary, the pea and bean plants which were kept in the dark continuously destroyed the IAA at a slower rate during the first 12-hour period. Since the same amount of IAA-1-C^{14} was used in the treatment of plants in all three experiments, the difference in the accumulated activity as CO_2 during a 30-hour period should provide a good indication that a species difference on the rate of destruction of IAA might exist. The specific activity of the respiratory CO_2 during illumination reached a maximum in approximately 4 hours in pea and bean plants, and in 8 hours in corn plants. The considerably higher specific activity of respiratory CO_2 in corn plants, as compared to those from pea and bean plants, suggests that a different pathway of IAA metabolism may exist in different plant species or possibly a different rate of destruction. This also indicates that the destruction of IAA by plants in vivo was dependent on light. If the biosynthesis of IAA in plants is not light dependent, then the concentration of this chemical will generally be

higher at nighttime; therefore, more growth of plants in nighttime can be expected.

The results on the effect of 2,4-D on the rate of destruction of IAA in bean plants are shown in tables II and III. The total recovery of radioactive carbon was low in these two experiments (carried out during April and May) as compared to the previous one (carried out during January), indicating that factors such as temperature, light intensity and photoperiod may affect the rate of IAA destruction. As indicated in table II, in the first groups which received no 2,4-D treatment, the recovery of radioactivity declined from 28.4 % in the third-day sample to 8.9 % in the seventeenth-day sample. These data indicate that almost three-fourths of the applied IAA was metabolized in the first three days. In the case of 2,4-D treated plants, the total recovery of C^{14} was consistently higher than that of the corresponding untreated plants. However, the difference in effect of 2,4-D between three levels used (5, 10, and 20 μgm) is not significant. Table III shows the results from the second experiment in which the dosages of 2,4-D used in the treatment were changed to 1, 5, and 50 μgm, respectively. No inhibitive effect was found in plants receiving 1 μgm of 2,4-D, as the total recoveries of radioactivity were practically identical as compared to that of the corresponding control groups. In the case of 5 μgm 2,4-D, the total recoveries of radioactivity were significantly higher than in the control group. The destruction of IAA was greatly reduced in the group which received 50 μgm of 2,4-D during the entire experimental period.

The effect of 2,4-D on the destruction of IAA-1-C^{14} in bean and corn plants as measured from the accumulated activity of respiratory CO_2 is shown in figure 3. The inhibitive effect of 2,4-D treatment again was demonstrated on the destruction of IAA in both bean and corn plants because the radioactivity in the respiratory CO_2 was always found to be less when 2,4-D was applied. In the case of bean plants, the significant effects induced by 2,4-D were observed after 4 hours of treatment, while in corn plants the inhibitive effect was not observed until the 12th hour. The production of CO_2 was greatly increased and the radioactivity was decreased in both 2,4-D treated plants, as indicated by the specific activity curves (fig 3). This result agrees with the data from tissue slice studies (3, 9) that at low concentration 2,4-D stimulates rate of respiration.

In the study of the relationship of 2,4-D to the endogenous auxin of the plant, Weintraub (11) found that the auxin content of 2,4-D treated bean buds was very much smaller than that of the normal buds. This difference is increased by increasing the amount of 2,4-D applied. They suggested that the lower auxin content is either due to a diminished rate of production or an augmented rate of destruction. The inhibitive effect of 2,4-D on the oxidation of exogenous IAA in both bean and corn plants, as found in these experiments, would suggest that the mechanism of action of 2,4-D which brings about a diminished content of extractable auxin in Weintraub's experiment is probably due to a diminished rate of production. It seems likely that the action of 2,4-D on exogenous IAA is probably due to an indirect effect possibly inhibiting the synthesis of an enzyme or other co-factors concerned with destruction of IAA. It is also possible that 2,4-D may stimulate the re-utilization of radioactive carbon from IAA-1-C^{14} and lead to the same end result. However, chromatographic study of alcohol extracts from leaf tissues receiving IAA-1-C^{14} alone or IAA-1-C^{14} plus non-radioactive 2,4-D revealed that both extracts contain six identical radioactive compounds. Therefore, it is doubtful if the lower production of respiratory $C^{14}O_2$ in the 2,4-D treated plants is due to re-utilization of radioactive carbon. Nevertheless, as a major hypothesis the selective toxicity of 2,4-D against broadleafed plants can no longer be explained simply on the basis of the disturbance of IAA metabolism in plants.

Summary

Indoleacetic acid-1-C^{14} with an activity of 6.50×10^7 cpm/mM has been synthesized.

The rate of the absorption and translocation of C^{14} after receiving IAA-1-C^{14} treatment on the primary leaf was studied in bean plants. Only 10 to 14 % of the C^{14} activity was absorbed and transported to the other parts during 14 days experimental period. A larger portion remained in the leaf which received IAA treatment.

The effect of light on the destruction of IAA-1-C^{14} was studied in kidney bean, pea and corn plants. The destruction of IAA-1-C^{14} was greatly inhibited in the dark as measured by the production of respiratory $C^{14}O_2$. The bean and pea plants destroyed IAA but at a slower rate during the first 12-hour period in the dark, while the destruction of IAA in the corn plants was completely light dependent.

The effect of 2,4-D on the destruction of exogenous IAA has also been demonstrated in the bean and corn plants. 2,4-D treatment decreased the rate of destruction of IAA in both plant species.

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ESTERIFICATION OF PHOSPHATE IN RIPENING FRUIT[1]

JOY D. MARKS,[2] ROBERT BERNLOHR[2] AND J. E. VARNER
DEPARTMENT OF BIOCHEMISTRY, COLLEGE OF AGRICULTURE, THE OHIO STATE UNIVERSITY, COLUMBUS 10, OHIO

The biochemical evidences of changes which mark the physiological development of fruit are associated with distinct periods in the life-history of the fruit. These periods are: 1) cell division; 2) cell enlargement; 3) maturation; 4) "autogenous climacteric"; and 5) senescence. The climacteric (4) refers to a respiratory pattern observed in many fruit following maturation, which is characterized by a sudden, sharp increase in respiratory rate to the "climacteric maximum." This maximum is reached by some kinds of fruit, while attached to the plant, or by other fruit as a post harvest phenomenon. The period of senescence, which terminates the development of all fruits, precedes final deterioration and decay of the natural product.

Robertson and Turner (7) proposed that the occurrence of limited respiration during the pre-climacteric period of fruit development may be due to the availability of phosphate acceptors. Thus, these workers implicated a controlling role for the process of oxidative phosphorylation during the later periods of fruit ripening. Pearson and Robertson (6) and Millerd, Bonner, and Biale (5) demonstrated that 2,4-dinitrophenol (DNP), an uncoupler of oxidative phosphorylation, increased respiration in pre-climacteric fruit (apple slices and avocado slices), but had no effect on fruit at climacteric and post climacteric stages. Analogous to the effect of DNP on avocado slices is the effect of naturally occurring uncouplers isolated from climacteric avocados by Millerd et al (5). These substances depressed P/O ratios of isolated mitochondria, and caused a corresponding increase in respiration. In view of these effects on respiration in both pre-climacteric fruit and isolated mitochondria, the hypothesis was proposed (5) that fruit ripening results from an uncoupling of oxidative phosphorylation.

In view of the evidence cited above, it was anticipated that a measurement of the status of phosphate esterification in intact fruit during the sequence of stages in its life-history would show whether a loss of phosphorylative capacity did, in fact, precede ripening. The ripening process is considered, in this paper, to embrace the periods of climacteric and senescence.

Measurements of the incorporation of radioactive phosphate into organic phosphate esters has shown significant esterification by tomato fruit during maturation, post climacteric, and senescent periods. It was possible to prevent phosphorylation by the uncoupling action of dinitrophenol, but complete disappearance of phosphorylative capacity did not occur naturally until the termination of senescence (apparent in tomato fruit from deep red skin coloration and the soft, structural condition of a completely ripened fruit). These findings, therefore, have established the occurrence of phosphorylation in whole fruit beyond the climacteric maximum of respiration and well into the post climacteric period of fruit ripening. Quantitative information concerning the phosphorylative capacity of tomato fruit are presented in this paper as a basis for the evaluation of the role of phosphorylation in the ripening processes of fruit.

MATERIALS AND METHODS

Tomato fruit were used for the experiments reported here. These were obtained from local gardens and from greenhouse plants (Ohio Wilt Resistant) grown under the supervision of the Ohio State University Department of Horticulture. Solutions used for the various treatments were injected into the locules of the tomatoes by means of a hypodermic syringe fitted with a two-inch needle. All incubations were at room temperature. Bruising injury was produced by rolling the fruit (under some mechanical pressure) prior to injection of labeled phosphate. Care was taken not to puncture the skin of the fruit during this treatment. After incubation, the fruit was sectioned and extracted by grinding in a mortar with sufficient trichloroacetic acid to bring the final concentration to 3 %. The clear supernatant fraction ob-

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[2] Charles F. Kettering Foundation predoctoral fellows.

tained by centrifugation was spotted on S and S No. 589 filter paper and the phosphorylated compounds chromatographed according to the procedure of Bandurski and Axelrod (2). Autoradiographs of these chromatograms were obtained on Eastman No-screen x-ray film. Quantitative determinations of the distribution of the labeled phosphates were made by cutting out the discrete spots indicated by the autoradiographs and assaying for radioactivity with an end window (1.4 mg/cm) Geiger-Müller tube and standard scaling circuit.

Experimental Results

Esterification Rate in Intact Fruit: Radioactive phosphate was injected into whole tomato fruit (ca 100 gm) at a rate of 0.5 ml phosphate (0.01 M) containing 100 microcuries of radioactivity. The uptake of inorganic phosphate proceeds rapidly, and reaches a steady state equilibrium in less than 24 hours (fig 1). All of the phosphate esters appear at about the same rate, and there is no observable tendency for the accumulation of large quantities of any single ester.

Phosphorylation Capacity of Intact Fruit: The capacity of tomato fruit for the incorporation of P^{32}-orthophosphate into organic esters was measured over the entire range of physiological ages. Size and skin color were a useful index of age, with climacteric respiratory behavior just preceding the onset of pink color in the fruit. Maximum esterification of P^{32}-phosphate was observed in green fruit, early in maturation, and likewise, was maximal in full-sized green, mature fruit at the "climacteric maximum" of respiration. The P^{32}-esterification curve for post climacteric fruit (fig 2) parallels the sharp decline in respiration

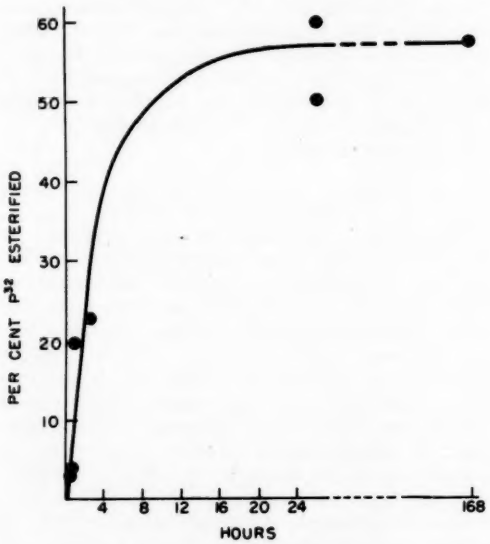

Fig. 1. Rate of phosphate esterification in whole fruit.

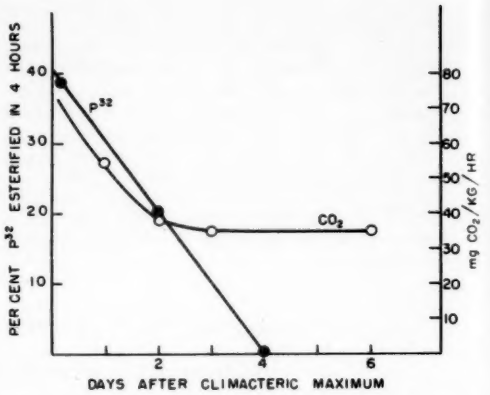

Fig. 2. P^{32} esterification and respiration (CO_2 evolution) of post climacteric tomato fruit. CO_2 data from Alban (1).

(CO_2 evolution) during the early post climacteric period. It would appear, therefore, that the early post climacteric decline in phosphorylative capacity is not the result of an uncoupling of oxidative phosphorylation. However, in late post climacteric stages, phosphorylative capacity completely disappears.

Sensitivity of Phosphorylation to DNP: A complete disappearance of phosphorylation pattern occurs when DNP, sufficient to produce a concentration of 10^{-4} M in the treated portion, is injected with P^{32}-orthophosphate into whole tomato fruit. The typical phosphorylation pattern shown in figure 3 A shows a phosphate ester component which moves at 54 % of the rate of inorganic phosphate (methanol–formic acid–water (2)). This has been tentatively identified as glucose-6-phosphate by co-chromatography with authentic glucose-6-phosphate in several solvents, but no attempt has been made to identify the other components. Figure 3 B shows the pattern obtained after DNP-treatment. No P^{32} component other than orthophosphate can be detected in the extracts of DNP-treated green or pink-green fruit, or in extracts of fully ripe fruit injected with P^{32}-orthophosphate alone (fig 3 C). The complete absence of any component in the TCA extract of DNP-treated fruit which could be attributed to substrate level phosphorylations probably indicates a rapid utilization of such esters which prevents their detection by the procedures used.

Effect of Bruising Injury on Phosphorylation Capacity of Both Pre-climacteric and Climacteric Green Tomato Fruit: A loss of phosphorylation capacity can also be induced by bruising injury (fig 4). The loss may be partial or complete, depending upon the severity of the bruise and the time elapsed after bruising. Complete loss of phosphorylative capacity occurs in a few hours, even though the bruised area is restricted to one part of the fruit.

Loss of Phosphorylative Capacity after DNP-Treatment: Inasmuch as fruit injected simultaneously with P^{32}-phosphate and DNP produced no de-

tectable organic phosphate ester, several green, mature fruit were labeled initially by injecting with P^{32}-phosphate and incubating for three days. DNP was then injected into one locule of each fruit, and the fruit extracted after 1, 4 and 24 hours to measure the rate of disappearance of P^{32} esters. Separate extracts were made for the tissue in direct contact with the DNP added and that tissue which was not in contact with DNP. The rate of disappearance of esters was identical in both extracts (fig 5). It appears, therefore, that bruising injury and DNP treatment are similar in that both cause a loss of phosphorylative capacity throughout the entire fruit even though the treatments are localized. These data indicate that both DNP and bruising bring about the release (or production and release) of a diffusible substance which results in the uncoupling of oxidative phosphorylation. These effects are summarized in figure 6.

Effect of DNP on Normal Ripening: In view of the uncoupling action of dinitrophenol on the phosphorylation of green, mature fruit, it became of interest to determine the character of ripening in a DNP-treated fruit. If the observed incorporation of P^{32}-phosphate into esters provides a source of energy required to carry out the ripening process, including degradation of chlorophyl and formation of red pigments (lycopenes), a green fruit uncoupled with DNP would be expected to remain green indefinitely. Green mature fruit were harvested, injected with DNP, and allowed to incubate for several weeks at room temperature. Control fruit ripened normally, during this period and developed the deep red color of fully ripe fruit. In contrast, DNP-treated fruit were essentially unchanged in appearance from the green, freshly harvested product. The requirement for an energy source, in the form of oxidative phosphorylation, would appear, therefore, to be a necessary factor for the successful achievement of "ripening."

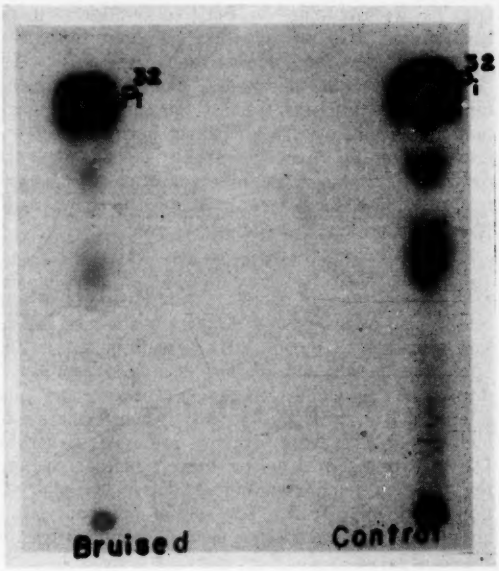

Fig. 4. Autoradiograph of chromatogram of TCA extracts of green mature tomato fruit. *Left.* Extract of bruised fruit, injected with P^{32}-orthophosphate. *Right.* Extract of normal fruit, injected with P^{32}-orthophosphate.

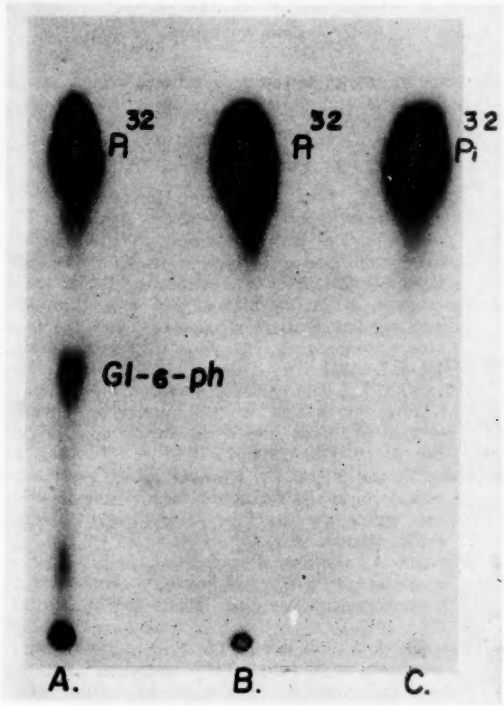

Fig. 3. Autoradiograph of a paper chromatogram of TCA extracts of tomato fruit. A. Extract of green, mature tomato fruit, injected with P^{32}-orthophosphate. B. Extract of green, mature tomato fruit, injected with P^{32}-orthophosphate plus DNP. C. Extract of fully ripe, tomato fruit, injected with P^{32}-orthophosphate.

Discussion

The concept of the ripening process in fruit which emerges from these studies is one of dynamic processes continuing through a major part of the post climacteric decline. Thus, the senescing fruit actively produces energy (as phosphate esters) and presumably is still carrying on biochemical work and biochemical syntheses. The correlation, made by previous workers, of respiratory increases and natural uncoupling would be invalidated by the existence of dinitrophenol-sensitive phosphorylation at climacteric maximum. Rather, the suggestion advanced by Hulme (3), that climacteric may be induced by increased availability of ADP provided by systems synthesizing new protein (enzymes), would be more consistent with our observations. Further evidence of an efficient oxidative phosphorylation system in climacteric

fruit has been obtained by Biale (unpublished) in recent studies with mitochondria prepared from avocado in different physiological stages.

Inasmuch as the inhibition of phosphorylation with dinitrophenol prevents fruit at the climacteric maximum of respiration from ripening normally, the energy required for ripening probably depends upon the oxidative phosphorylation observed in post climacteric fruit. As ripening is completed, phosphorylative capacity can no longer be detected.

Summary

The occurrence of phosphate esterification has been established, by means of P^{32} tracer techniques, for intact tomato fruit during early periods in maturation (pre-climacteric), and likewise has been established during the post climacteric period of senescence. The failure of fruit to ripen normally, when formation of high-energy phosphate esters is inhibited, either by DNP-treatment or bruising injury, further sustains the apparent requirement for oxidative phosphorylation by ripening fruit. The role of phosphorylation in fruit ripening would thus seem to point toward the existence of energy requiring syntheses, which are essential to the completion of ripening by senescing fruit.

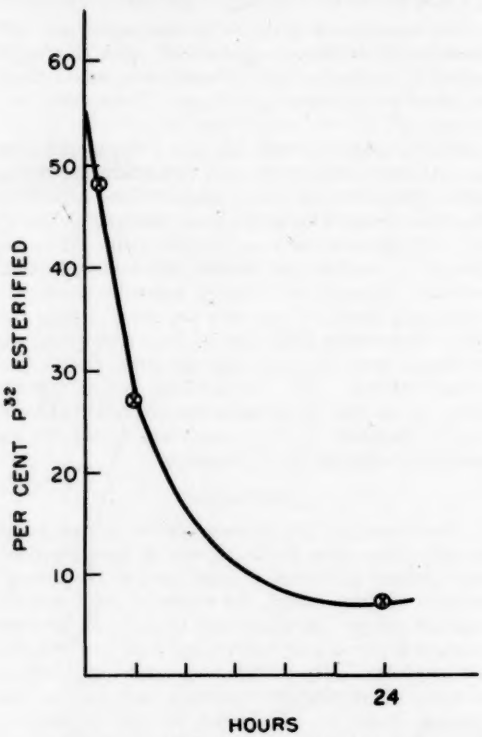

Fig. 5. Rate of disappearance of P^{32}-phosphate esters after DNP-treatment. Circles indicate rate in portions of fruit in direct contact with injected DNP. Crosses indicate rate in portions which contained no visible traces of injected DNP.

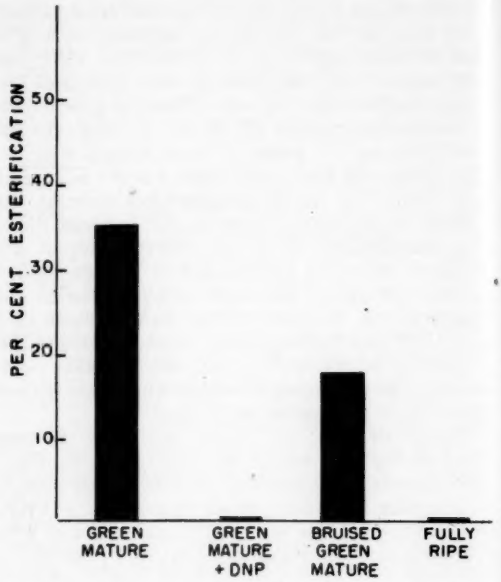

Fig. 6. Comparison of phosphorylation capacity by tomato fruit under various conditions.

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CONVERSION OF TRYPTOPHAN-2-C^{14} TO INDOLEACETIC ACID BY WATERMELON TISSUE SLICES[1,2]

WARREN N. DANNENBURG[3] AND JAMES L. LIVERMAN

DEPARTMENT OF BIOCHEMISTRY AND NUTRITION, TEXAS AGRICULTURAL EXPERIMENT STATION, COLLEGE STATION, TEXAS

It was suggested some years ago that the plant growth hormone, IAA[4] arises from TTP through a series of reactions involving IPyA or TNH_2 and IAc (13, 21). Subsequently, when IAN was isolated from natural sources, it was proposed that IAN and IAM (14) were likely intermediates in the conversion of TTP to IAA (11). This latter view received additional support with the demonstration that IAN can be converted to IAA (19). Recently, however, IAM was demonstrated in the presence of respiring tissue when exogenous IAA was added and this suggests that IAM arises from IAA rather than being an intermediate in the conversion of TTP to IAA (7). Still other investigators present evidence suggesting that IPyA and TNH_2 are not intermediates in the conversion of TTP and IAA by the mung bean (8, 22).

Experiments carried out in this laboratory in which DL-TTP-2-C^{14} was incubated with watermelon tissue slices indicate that TTP can serve as a precursor of IAA in this tissue and furthermore the data suggest that this pathway is through IPyA.

MATERIALS AND METHODS

The watermelon seed used in these experiments were of the New Hampshire Midget variety No. 879 obtained from the Farmer Seed and Nursery Company, Faribault, Minnesota. The seeds were germinated and the plants were grown under artificial light in sand culture watered with nutrient solution (17). Approximately 7 to 10 days after hand pollination, when the melons were approximately 3 cm long, the outer covering (rind) was removed, and the remaining tissue sliced into thin sections. These sections were randomized and slices equivalent to two melons (10 to 12 gm) were added to a 125-ml Erlenmeyer flask containing 9 ml of M/15 KH_2PO_4. Each ml of buffer contained 300 units of penicillin G and 5 mg of DL-TTP (specific activity equal to 1,032,000 cpm/mg). The medium was made 1×10^{-3} M with respect to ascorbic acid to inhibit the destruction of IAA (2, 23). The final pH was 4.5 ± 0.1.

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[2] This work was supported in part by grants from the Atomic Energy Commission and the National Science Foundation to Dr. J. L. Liverman.

[3] Part of the material here is to be submitted to the Graduate School of Texas A. & M. College in partial fulfillment of the requirements for the Ph.D. degree.

[4] IAA (indoleacetic acid); IPyA (indolepyruvic acid); IGA (indoleglycolic acid); IAH (indolealdehyde); IAM (indoleacetamide); IAN (indoleacetonitrile); IAAP (indoleacetylaspartic acid); TTP (tryptophan); TNH_2 (tryptamine); IAc (indoleacetaldehyde); IOA (indoleglyoxylic acid); TOH (tryptophol); 5-OH TTP (5-hydroxytryptophan).

The optimum pH for the conversion of TTP to IAA by spinach tissues (25) has been reported to be 7.5, but it is also at this pH that IAA is most rapidly destroyed (20). It was decided therefore to use pH 4.5 to retard this enzymatic destruction, and even though the amounts of TTP converted would probably be decreased, the radioactive label would enable detection of the indole derivatives. In addition, the acid pH would discourage bacterial growth and enhance the stability of IPyA (1). The flasks were plugged with cotton, wrapped in black, light-proof paper and placed on a shaker at room temperature ($25 \pm 2°$ C) for 20 hours.

In more recent experiments, where TTP-2-C^{14} was incubated with Avena coleoptile tissue for 20 hours, compounds apparently identical to those found in watermelon were observed. Further experiments with acetone dried powder of Avena showed these same compounds to be formed after a 30-minute incubation period (unpublished, Dannenburg and Liverman). It appears then that the conversion of TTP to IAA in these experiments with watermelon tissue constitutes a normal biochemical conversion. After incubation, the flasks were placed in boiling water for 10 minutes and then cooled. The contents and wash water from each flask were placed in a mortar and triturated with washed, ignited sand. The resulting brei was centrifuged, the supernatant was decanted, and then partitioned into anhydrous, peroxide-free ether at pH 8.1 and 2.8, respectively.

PRELIMINARY EXTRACTION EXPERIMENTS: A clear chromatographic separation of the indole compounds was not possible without previous treatment because of the proximity of the neutral and basic substances in our solvent system. Success with a preliminary solvent extraction procedure for separating TNH_2, IAM and IAN was so promising that a mixture of several know acidic, basic, and neutral indole compounds were partitioned as discussed below prior to attempting a separation of the products of TTP metabolism by paper chromatography (fig 1). The chemical separation was done to obtain information as to the type of compounds present as far as solubility is related to structure and to separate the basic substances from the neutral substances. Table I shows that this preliminary partitioning with solvents results in a clean separation of all compound except the IAM which is found in both the neutral and the basic fractions. The presence of IAM in both the basic and neutral fraction was not expected since amides are generally insoluble in 5 % HCl (16).

Since the indole nucleus is known to be labile in the presence of strong mineral acids (18), an experiment was set up to determine the rate of destruc-

tion of the indole ring by 5 % HCl. The indole ring is more stable towards mineral acid than the pyrrole ring to which the reaction is attributed (12). One mg of indole was dissolved in 20 ml of 5 % HCl with the aid of ethanol and placed on a shaker. Aliquots were taken at intervals and the amount of indole determined (5). Table II shows there is a rapid destruction of indole with time, but the amount of destruction is small after the first 15 minutes which approximates the time the indole compounds are in contact with the 5 % HCl. This destruction is approximately 40 %. Though this amount of destruction does not interfere with a qualitative test for the compounds, it would discourage the quantitative aspects of the extraction procedure.

Basic and Neutral Fractions: As shown in figure 1, the aqueous solution (approx. 50 ml) was adjusted to pH 8.1 with Na_2CO_3 and partitioned three

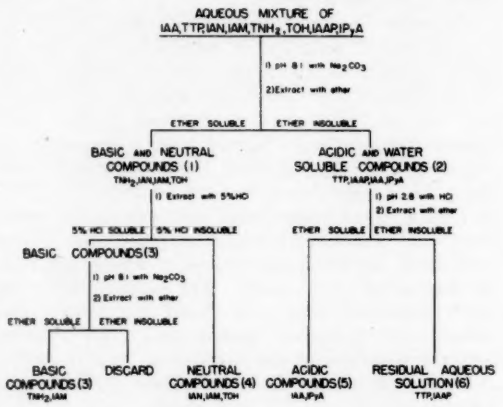

Fig. 1. Partitioning of an aqueous solution of known indole compounds into the residual aqueous solution, neutral, basic and acidic fractions.

times into 20-ml portions of ether. The ether extracts (I) containing the basic and neutral compounds were combined and then further resolved by partitioning the basic compounds into 5 % HCl, leaving the neutral compounds in the ether fraction (IV). The pH of the aqueous 5 % HCl extract was adjusted to 8.1 with Na_2CO_3 and the basic components were extracted back into ether with three 20-ml portions (III). The aqueous portion contained no Ehrlich positive material and in experiments with the tissue it was discarded, since it had no radioactivity. The two ether extracts containing the neutral and basic substances were washed with a saturated solution of NaCl, dried with anhydrous Na_2SO_4, filtered, and evaporated to dryness under reduced pressure without heat. The residue of each fraction was resuspended in 1 ml of anhydrous peroxide-free ether.

Acidic Fraction: The aqueous solution (II) remaining after removal of the basic and neutral substances was adjusted to pH 2.8 with HCl and extracted with three 20-ml portions of ether. The ether extracts (V) were combined, washed with a saturated solution of NaCl, dried with anhydrous Na_2SO_4, filtered and taken to dryness under reduced pressure without heat. This residue was then resuspended in 1 ml of ether.

Residual Aqueous Solution: The residual aqueous solution (VI) remaining after the removal of the basic, neutral, and acidic compounds was lyophilized and resuspended in one ml of water.

Chromatography: The acidic, basic, and neutral fractions were streaked separately along a one-inch line of Whatman 3 MM paper, with the exception of the aqueous sample where only 0.5 ml was applied to the paper. The chromatograms were equilibrated for 12 hours in the chromatographic chamber before being placed in the solvent of isopropanol : NH_4OH : H_2O (8 : 1 : 1, v/v) for ascending development. In addition to making these one dimensional chromatograms two dimensional chromatograms were made on all fractions except the residual aqueous solution. The developing solvent for the acidic and basic frac-

Table I

The Distribution of IAA, TTP, IAN, IAM, TNH_2, IPyA, TOH, IAAP When Partitioned According to the Described System

Compound in mixture	Fraction indole compound found			
	Acidic	Basic	Neutral	Residual aqueous
Indoleacetylaspartic acid (IAAP)*	...	...	...	+++
Indoleacetic acid (IAA)	+++	...	...	...
Indolepyruvic acid (IPyA)*	+++	...	...	...
Tryptophan (TTP)	...	...	...	+++
Tryptophol (TOH)*	...	...	+++	...
Indoleacetonitrile (IAN)	...	...	+++	...
Indoleacetamide (IAM)	...	+	++	...
Tryptamine (TNH_2)	...	+++	...	...

+++ Compound only in this fraction.
++ Majority of compound in this fraction.
+ Indicates trace amount found.
* Not partitioned as mixture, but separately.

Table II

The Effect of 5 % Hydrochloric Acid on the Destruction of the Indole Nucleus

Min	% Indole recovered
3	76.5
6	68.3
9	62.2
15	59.7
20	54.6
25	54.6
35	53.8
45	58.0
60	52.7

TABLE III

R_f VALUES OF INDOLE DERIVATIVES OBTAINED BY INCUBATION OF WATERMELON TISSUE SLICES WITH TRYPTOPHAN-2-C^{14} AND DEVELOPED ON WHATMAN 3 MM PAPER WITH ISOPROPANOL : NH_4OH : H_2O AS THE DEVELOPING SOLVENT

FRACTION OR COMPOUND **	R_f VALUES (AV) *	R_f VALUES CALCULATED FROM		COLOR WITH EHRLICH'S REAGENT	R_f VALUE OF UNKNOWN OVER-SPOTTED WITH KNOWN COMPOUND	PROBABLE COMPOUND **
		RADIOACTIVITY	COLOR			
Acidic	0.17	+	...	.....	...	IPyA
	0.38	+	...	.....	...	Unidentified
	0.49	+	+	Pink	0.49	IAA
	0.65	+	+	Pink	...	IGA †
	0.81	...	+	Pink	0.84	IAH
Basic	0.81	+	...	.....	...	Unidentified
	0.89	+	...	.....	0.85	TNH_2
Neutral	0.81	+	+	Blue	...	Unidentified
	0.96	+	...	.....	0.95	IAN
	0.99	+	+	Purple	0.99	Unidentified
Residual aqueous solution	0.07	+	...	.....	...	IAAP
	0.15	+	...	.....	...	5-OH TTP
	0.40	+	+	Purple	0.40	TTP
	0.50	+	+	Light blue	...	Unidentified
	0.71	+	+	Light blue	...	Unidentified
IAA	0.48	...	+	Pink	...	IAA
IPyA	0.17	...	+	Yellow-green	...	IPyA
IGA	0.61 (0.64) †	...	+	Pink	...	IGA
IAH	0.84	...	+	Pink	...	IAH
IAM	0.79	...	+	Purple	...	IAM
TNH_2	0.85	...	+	Blue	...	TNH_2
IAN	0.95	...	+	Purple	...	IAN
IAAP	0.073	...	+	Purple	...	IAAP
TTP	0.40	...	+	Purple	...	TTP
5-OH TTP	0.17	...	+	Purple	...	5-OH TTP

* These values represent the averages of several determinations.
** See text for abbreviations.
† R_f value taken from Fischer, Planta 43: 288–314. 1954. Identification is tentative.

tion in the first direction was isopropanol : acetic acid : H_2O (6 ml acetic acid/100 ml of 80 % isopropanol); in the second direction the above isopropanol : NH_4OH : H_2O solvent was used. The neutral fraction was developed with distilled water in the first direction, while isopropanol : NH_4OH : H_2O was used in the second direction. The R_f values obtained with the known indole compounds for both one and two dimensional chromatography are shown in tables III and IV.

RADIOACTIVITY MEASUREMENTS: In experiments with tissue incubated in the presence of TTP-2-C^{14}, the same procedure was used as for the separation of known compounds, with the exception that soluble proteins which still remained were precipitated with boiling ethanol. After drying the paper chromatograms and spraying with Ehrlich's reagent, they were strip counted on a Nuclear D-47 gas flow counter and subsequently were used for the preparation of radioautograms. Two dimensional chromatograms were not strip counted, but only subjected to radioautography.

EXPERIMENTAL RESULTS

Two types of controls were used with the incubation mixture, one was the tissue mixture without added TTP, and the second was with a heated tissue incubation mixture containing TTP. Chromatograms of extracts of the first control did not contain any detectable Ehrlich positive compounds. Strip counting or radioautograms of extracts of the heated tissue were negative except for the TTP. This shows that the tissue without added TTP or heat inactivated tissue with TTP does not produce indole compounds in amounts detectable by Ehrlich's reagent or radioactivity. In addition, the solution mixtures in the flasks after incubation were clear and the tissue was essentially the same color as it was when placed into the flasks. Since the controls did not show any of the indole compounds detected in experimental flasks, it is assumed that the conversion of TTP to IAA or its intermediates were due to the tissue per se and not due to contamination by microorganisms or to non-enzymatic conversion.

The results of these experiments using one and two dimensional chromatograms are summarized in tables III and IV while the radioautograms for one dimensional chromatograms are shown in figure 2.

ACIDIC COMPOUNDS: Five compounds were detected in the acidic fraction by strip counting, by radioautography, and by the use of Ehrlich's reagent. Two of the compounds have been tentatively identi-

fied as IAA and IPyA. The R_f values of the compounds suspected of being IAA and IPyA were calculated from the radioactive peak of the strip count and from the radioautograms. The calculated R_f values agreed with those determined with known IAA and IPyA. Additional evidence that one of the radioactive spots was IAA was obtained by overspotting an aliquot of the unknown fraction with known IAA and chromatographing. The new spot after movement, as detected by Ehrlich's reagent, coincided with the radioactivity and agreed with the R_f value found for IAA chromatographed alone. The identification of IPyA was made by comparing the R_f value of the unknown with synthetic IPyA (3). This unknown acidic compound and IPyA gave a yellow-green color with Ehrlich's reagent and showed a yellow fluorescence with a dark purple background when viewed under ultraviolet light after spraying with 1,2-diamino-4-nitrobenzene (10). This reagent is relatively specific for keto groups. The spot identified as IAA did not react with this reagent.

It has been reported that IPyA is unstable (19, 25) and more recently Bentley and co-workers reported that it is completely destroyed when chromatographed in basic solvent systems (1). The results of experiments reported here confirm these results in all respects except that our experiments show that there is not a complete destruction of IPyA chromatographed in a basic buffered solvent system. Several spots were found upon chromatographing the synthetic IPyA, but the amount of decomposition could be decreased to only three spots by chromatographing and drying in an atmosphere of nitrogen. In addition to the spot identified as IPyA, one spot was identified as IAA while a third spot which migrated below IAA was not identified. The R_f values of these compounds were 0.17, 0.45, and 0.36, respectively.

The acidic compounds moving ahead of IAA (R_f 0.65 and 0.81) are probably decomposition products of IAA. An explanation for these observations may be that IAA arises from exposure to normal room light and air during the 12-hour equilibration and 5- to 6-hour developing time since IAA is photolabile and decomposes when chromatographed on paper (15). The light mediated decomposition apparently results in the formation of IGA and IAH (4). Although our developing solvent is different with regard to the amount of NH_4OH present, the R_f values of the unknown compounds found in our experiments agree with the R_f values reported for IGA and IAH (4). Experiments have demonstrated (19) that the minimum concentration of NH_4OH in this solvent system is critical in order to obtain comparable R_f values of indole compounds. The concentration of

TABLE IV

R_f VALUES OBTAINED ON TWO DIMENSIONAL CHROMATOGRAPHY OF INDOLE COMPOUNDS ON WHATMAN 3 MM PAPER

FRACTION OR COMPOUND *	R_f VALUE SOLVENT **			R_f VALUES CALCULATED FROM		PROBABLE COMPOUND
	A	B	C	RADIOACTIVITY	COLOR	
Acidic	0.76	0.18	...	+	...	IPyA
	0.67	0.39	...	+	...	Unidentified
	0.82	0.49	...	+	...	IAA
	0.84	0.68	...	+	...	IGA †
	0.78	0.80	...	+	+	IAH
Basic	0.78	0.79	...	+	...	TNH_2
	0.88	0.79	...	+	...	Unidentified
Neutral	...	0.92	0.43	+	...	IAN
	...	0.95	0	+	...	Unidentified
	...	0.93	0.54	+	...	Unidentified
IPyA	0.79	0.18	...	...	+	IPyA
	0.81	0.37	...	...	+	Unidentified
	0.87	0.50	...	...	+	IAA
IAA	0.84	0.48	...	...	+	IAA
	0.82	0.63	...	...	+	IGA †
	0.83	0.81	...	...	+	IAH
IAN	...	0.91	0.44	...	+	IAN
IAM	0.80	0.78	0.58	...	+	IAM
TNH_2	0.76	0.80	...	...	+	TNH_2
TTP	0.37	0.38	...	...	+	TTP

* For abbreviations, see text.
** Solvent A, isopropanol : HOAc : H_2O. Solvent B, isopropanol : NH_4OH : H_2O. Solvent C, distilled water.
† Identification of this compound is tentative.

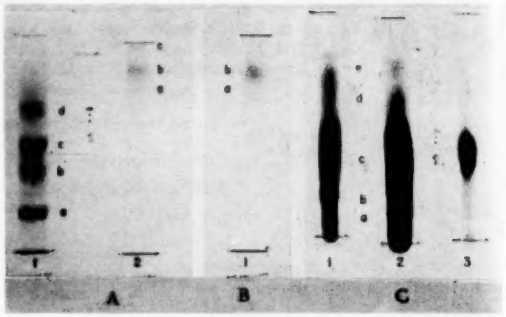

FIG. 2. Radioautograms of the acidic, neutral, basic, residual aqueous solution, and extract of brei, obtained by incubating watermelon tissue slices in tryptophan-2-C^{14} and chromatographed on Whatman 3 MM paper using isopropanol : NH_4OH : H_2O (8 : 1 : 1) as the developing solvent.

Section A-1, Acidic Fraction: spot a, indolepyruvic acid; b, unidentified; c, indoleacetic acid; d, indoleglycolic acid (tentative identification).

Section A-2, Neutral Fraction: spot a, unidentified; b, indoleacetonitrile; c, unidentified.

Section B-1, Basic Fraction: spot a, unidentified; b, tryptamine.

Section C-1, and C-2, Residual Aqueous Fraction: spot a, indoleacetylaspartic acid (see text); b, 5-OH TTP; c, tryptophan; d, unidentified; e, unidentified.

Section C-3, Extract of Brei: spot c, tryptophan.

NH_4OH in the solvent system of isopropanol : NH_4OH : H_2O (80 : 5 : 15, v/v) is above this critical concentration, and consequently, the R_f values obtained in the two systems are comparable. The presence of more than one spot due to ionization was eliminated by using an adequately buffered solvent system (19). These results do not agree with those of Bentley et al (1) and since our identification of IGA is indirect depending upon R_f values obtained by other workers (4), this identification can only be tentative. When high concentrations of IAA (0.1 to 0.2 mg) were chromatographed under the conditions of our experiments, these same spots are detected. The spots are not obtained when lower concentrations of IAA (0.001 to 0.01 mg) are used.

The identity of the spot with an R_f value of 0.38 which travels below IAA is unknown. It was detected by its radioactivity. It is not TTP as shown by the results of two dimensional chromatography (table IV). This evidence coupled with the fact that TTP has never been observed in the acidic fraction when using the described method of solvent partition is additional evidence that the compound is not TTP. A compound with a similar R_f value has been reported and it was observed in our experiments that synthetic IPyA decomposed to give a spot with a R_f value similar to that of the reported compound (7). The results obtained with a two dimensional chromatogram agreed with those found on a one dimensional chromatogram as shown in table IV. Recent evidence is not in agreement with this observation, and the data obtained by other workers (1) indicate that the spot arising from IPyA with R_f 0.38 is IGA.

Basic Compounds: Two compounds are chromatographically detected in the basic fraction (fig 2 B). One of these compounds has been identified as TNH_2, while the other remains unidentified. The R_f value of the unknown agrees well with known TNH_2 in one and two dimensional chromatography (tables III and IV). When the basic fraction was overspotted with known TNH_2 and chromatographed in one dimension, the known as detected with Ehrlich's reagent coincided with the radioactivity. The unidentified compound has the same R_f as IAM when developed in isopropanol : NH_4OH : H_2O, but two dimensional chromatography using isopropanol : HOAc : H_2O in addition to the basic buffered solvent shows that this compound cannot be IAM. Its identity is unknown.

Neutral Compounds: Three components were found in the neutral fraction (fig 2 A). One of the compounds was identified as IAN by one and two dimensional chromatography (tables III and IV), and overspotting with known IAN and developing in one dimension demonstrated that the spot as detected with Ehrlich's reagent coincided with the radioactivity. As in the basic fraction, one of the compounds had the same R_f as IAM when developed in a basic solvent system, but two dimensional development employing water in addition to the basic solvent system indicated that this compound was not IAM. On one dimensional chromatograms, it was not thought odd that a compound found in both basic and neutral fractions should have the same R_f value since known IAM partitioned in a similar manner.

In addition to the IAN and the unidentified spot of the neutral fraction, an Ehrlich positive radioactive spot was found which travelled with the solvent front when using isopropanol : NH_4OH : H_2O. A two dimensional chromatogram using water as the solvent in the first direction showed this compound to remain at the origin (table IV). A compound has been reported previously (4) which behaves similar to this compound, but it was not identified.

Residual Aqueous Compounds: The residual aqueous solution contained TTP, possibly indoleacetylaspartic acid (IAAP) (6), 5-OH TTP, and two other unidentified compounds (table III and fig 2 C). The unidentified compounds with R_f values of 0.50 and 0.71 colored a light blue with Ehrlich's reagent, while those with R_f values of 0.073 and 0.15 which showed no color were detected by their radioactivity. The compound identified as IAAP agrees with data obtained by other workers (6), but is contrary to their later publication (7) which stated that at pH 3.0, the IAAP was also found in the acid-ether extract. In an attempt to resolve this contradiction, an aqueous solution of known IAA and IAAP was partitioned into ether at pH 2.8. The results showed that the IAAP remains in the aqueous fraction, although if the water is not completely removed from the extracting ether with anhydrous Na_2SO_4 before paper chromatography IAAP will be found in the ether layer. These results would seem to reconcile the apparent discrepancy reported above. The identification of IAAP was based on published reports (6, 7), but recent evidence (communication, Norman Good) indicates that this compound is not a conjugate of IAA and aspartic acid, rather one of TTP and malonic acid.

The compound in the residual aqueous solution with an R_f of 0.15 has the same R_f value as known 5-OH TTP. Since this spot is ninhydrin positive, it has been tentatively identified as 5-OH TTP.

Since it is desirable to know if the aqueous extraction of the tissue was complete, the brei remaining after the described treatment was extracted with 95 % ethanol over a period of three days. Radioautograms indicated that the only radioactive compound remaining was TTP (fig 2 C).

Discussion

Numerous indole compounds have been detected by the techniques employed in the present study, but the sequence in the formation of IAA and TTP is still only speculative. The evidence presented in these experiments shows that IAA is formed from TTP and that during this biosynthesis several indole compounds are formed. Indole compounds are not detected with Ehrlich's reagent in the absence of added TTP, nor can they be detected by either radioactivity or Ehr-

lich's reagent in the presence of heat inactivated tissue. This confirms previous observations (9, 24, 25) that TTP acts as a precursor of IAA and that the process is enzymatic.

Since each fraction except the residual aqueous solution represented the same amount of tissue, the amount of radioactivity as seen by the darkening of the x-ray film should give an indication as to which compounds are most abundant in the formation of IAA, and consequently those which are involved in the biosynthetic pathway. Of all the compounds detected, IPyA contained the greatest radioactivity relative to the spot identified as IAA which showed only slightly less radioactivity. This was indicated by the relative degree of darkening of the x-ray film, and by the activity detected by strip counting. The results suggest that IPyA is probably one of the principal intermediates in the conversion of TTP to IAA and that the pathway previously postulated (21) is probably correct.

The data do not offer definite proof that the spot identified as IPyA is the principal intermediate, but all the data is certainly compatible with this view. It appears to contain the most radioactivity with a single exception. This apparent exception is found in the compound in the aqueous fraction identified as 5-OH TTP (fig 2 C, spot b). This fraction represents only one-half of the tissue, and the spot with an R_f of 0.15 appears to contain more radioactivity than that present in the IPyA of the acid fraction. The formation of a hydroxy derivative of TTP would not be inconsistent with the pathway going through either IPyA or TNH_2 as this could represent the first step in the oxidation of TTP. Since this compound does contain more radioactivity than IPyA or TNH_2 and since IPyA is more radioactive than TNH_2 it is reasonable to suspect it of being the precursor of both TNH_2 and IPyA. This finding gives additional evidence of IAA biosynthesis from TTP with IPyA being the principal intermediate.

Indolepyruvic acid chromatographed on Whatman 3 MM paper in isopropanol : NH_4OH : H_2O was detected with Ehrlich's reagent under the conditions of the present experiments. Our results confirm those of other workers (19, 25) which show that IPyA is unstable, although we find that the IPyA is not completely destroyed in basic solvent systems as reported by Bentley (1). At present the discrepancy cannot be explained unless possibly the solvent extraction removes impurities resulting in greater stability of the IPyA or there is greater stability due to chromatographing in a nitrogen atmosphere. The agreement of the R_f value obtained by color reaction and radioactivity seems to confirm the view (19) that isopropanol : NH_4OH : H_2O can be used as a developing solvent for IPyA, although an acidic buffered solvent system appears to result in greater stability of the IPyA.

It has been reported that the decomposition of IAA results in the formation of IGA and IAH (4). In our experiments TTP labeled in the 2 position of the side chain gives rise to radioactive IAA and nonradioactive IAH; and in the presence of TTP-3-C^{14}, both IAA and IAH are radioactive (unpublished, Dannenburg and Liverman). This would tend to confirm the suggestion that the TTP molecule remains intact during the conversion of TTP to IAA.

The R_f values obtained for two dimensional chromatography of one of the unknown acidic compounds agrees with those obtained for known IAA. Since the spot identified as IAA does not react with 1,2-diamino-4-nitrobenzene, a keto reagent it appears unlikely that the spot is IOA which has been reported to have the same R_f value as IAA (4).

The role of TNH_2 and IAN in the conversion of TTP to IAA is not well understood. It has been demonstrated that TNH_2 and IAN can be converted to IAA (19) and Gordon has shown that when the enzyme system capable of converting IAc to IAA is inactivated by x-irradiation that an alternate pathway does exist (8). Since both TNH_2 and IAN can apparently be formed in the presence of TTP as shown in these experiments, they may function as alternate pathways of IAA synthesis from TTP when the pathway through IAc is blocked.

The presence of a transoximase in plants has been demonstrated (26) and it is possible that IAN may be formed by the condensation of hydroxylamine with IAc forming an oxime which is then dehydrated to IAN. This seems particularly plausible in view of the recent evidence showing IAM formation in the presence of exogenous IAA (7).

Summary

1. A mixture of known indole compounds which may be intermediates in the conversion of TTP to IAA have been separated by solvent partition and paper chromatographic methods.

2. Evidence as to the conversion of labelled TTP to IAA by watermelon tissue slices has been presented with the detection of several radioactive indole compounds. Results indicate it to be a potentially useful tool in determining the biosynthetic pathway of IAA from TTP.

3. The main pathway in the conversion of TTP to IAA using watermelon tissue slices seems to be through IPyA based on the radioactivity of IPyA in relation to IAA.

4. Both TNH_2 and IAN are formed from TTP and it is possible that they function as alternate pathways for IAA formation when the pathway through IAc is blocked.

5. Indoleacetonitrile formation may be by the condensation of IAc with hydroxylamine to form an oxime with subsequent dehydration to IAN.

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Norman Good of Science Service Laboratory, London, Ontario. D,L-Tryptophan-2-C^{14} (0.00233 mc/mg) obtained from Tracerlab, Inc., Boston, Massachusetts.

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SEDOHEPTULOSE IN COLEUS[1]

N. E. TOLBERT, C. W. NYSTROM[2] AND P. C. KERR

Biology Division, Oak Ridge National Laboratory,[3] Oak Ridge, Tennessee

Large amounts of the seven-carbon sugar, sedoheptulose, are found in Crassulacean plants, particularly in Sedum (8, 9). A role for sedoheptulose phosphate has been demonstrated in the photosynthetic carbon cycle by Benson et al (2) and in the pentose phosphate cycle by Horecker and Mehler (5). A function for the free sugar remains obscure (11, 12). This paper presents data indicating that varieties of Coleus contain large amounts of free sedoheptulose and sedoheptulose phosphate, comparable to the amount in Sedum. In Coleus, the reservoir of sedoheptulose, to-

[1] Received November 29, 1956.

[2] Present address: Research Department, R. J. Reynolds Tobacco Company, Winston-Salem, North Carolina.

[3] Operated by Union Carbide Nuclear Company for the U. S. Atomic Energy Commission.

gether with sucrose, appears to function in carbohydrate storage. The rate of C^{14} labeling of free sedoheptulose and its total amount in the Coleus plant exhibit diurnal changes. Sedoheptulose phosphate does not show such variations.

Experimental

For experiments on diurnal variation, three varieties of *Coleus Blumei* Benth. were grown in a room artificially lighted with 500 to 1000 ft-c of light from daylight fluorescent and incandescent white lamps for 15 hours a day. Plants grown under these conditions were very green but those raised in the greenhouse in full sunlight were bleached and stunted. Free sedoheptulose was present in the leaves of plants grown under all environmental conditions.

The three varieties of Coleus that have been used extensively in this investigation have been deposited in the Herbarium of the University of Tennessee. One variety is pigmented yellow and has been identified as Golden Bedder. The second variety is of a variegated type, with or without anthocyanin in sharply defined regions, and is named Lincoln. The third variety has deeply serrated or toothed edges, but its varietal name has not been ascertained. It is similar but not identical to the variety known as Butterfly. For the purpose of this manuscript, it will be referred to as Coleus III.

Three to six days before an experiment, plants were exposed during a 15-hour day to 2200 to 2700 ft-c of white light from 150-watt General Electric reflector spot lamps cooled by water (14). Enough plants were so illuminated to provide leaves at the same stage of development for one experiment. The leaves were cut from the plant, quickly weighed, and their petioles placed in a small thimble of water in a photosynthetic exposure chamber, where they were left open to the air for 5 minutes. This procedure was used to prevent some of the photosynthetic induction effects during the night measurements, when the leaves were brought into the light for testing. The photosynthetic chamber consisted of a test tube (diameter, 30 mm; length, 80 mm) with a large ground glass cap containing a short sidearm. In a small boat in the sidearm was placed 25 μc of a dilute solution of $NaHC^{14}O_3$ (19 % C^{14}). The chamber was closed and the $C^{14}O_2$ was released when lactic acid was injected into the boat through a hypodermic needle pushed through a serum cap over the end of the sidearm. The exposure chamber was mounted in a bath whose temperature was adjusted so that a thermometer inside the chamber read 23° C. Light from a reflector lamp was directed on the exposure chamber through the glass sides of the water bath so as to deliver 1000 to 2000 ft-c to each side of the leaf.

The leaves were killed by two procedures. When they were to be used for quantitative sedoheptulose analysis, a leaf was dropped into 50 ml of boiling 85 % ethanol, and extracted two additional times with 25 ml of the same solution. The extracts were combined and diluted to 100 ml in a volumetric flask. Alcohol extraction was used since it would not remove the pentosans that would interfere in subsequent analyses of sedoheptulose. When $C^{14}O_2$ fixation experiments were run the leaves were first extracted in boiling 85 % alcohol as described above and total sedoheptulose could be analyzed on this extract. Further extraction of the leaf tissue with hot water removed between 20 to 35 % more C^{14} which, by chromatographic analysis, contained organic phosphates and malic acid, but no free sugars. Aliquots of the alcohol and water extracts were counted for total C^{14} fixed, and then they were combined and other aliquots chromatographed. Paper chromatograms were first developed in water-saturated phenol and then with butanol–propionic acid–water, 2 : 1.4 : 1 prepared according to Benson et al (1). Radioactivity was located by No-Screen X-ray film, and compounds were counted on the paper by use of a large Geiger tube. Data are expressed as percentage distribution in sedoheptulose of the total $C^{14}O_2$ fixed among the various alcohol and water soluble products.

Total sedoheptulose in the extract was estimated by two methods, sedoheptulosan being used as a standard: (a) semiquantitatively by paper chromatography, and (b) quantitatively by the orcinol-HCl procedure (4) applied to an aliquot of the original extract. The orcinol-HCl method gave good recovery, as indicated when extra sedoheptulosan was added; however, the absorption spectrum of the color after development indicated the presence of some interfering pigments which might introduce substantial error when the quantity of sedoheptulose was small. The absorption maximum of 580 to 620 mμ for sedoheptulose was usually shifted by 10 to 20 mμ to a wave length shorter than that obtained with crystalline sedoheptulosan (15). Large amounts of interfering substances in the extract formed a complex in orcinol-HCl with an absorption maximum around 425 mμ; this curve tailed into the sedoheptulose colored complex. Though fructose is known to react in this test to form a colored complex at about 425 mμ maximum, it could not have caused the interference, since paper chromatographic analyses indicated that it was not present in significant amounts in the Coleus leaves. Because of the apparent interferences by other components in the extract, all samples were decolorized at about pH 3 with very small amounts of Norite A before analysis for sedoheptulose. This treatment substantially reduced the interfering substances. The values for sedoheptulose content after charcoal treatment were about 20 % lower than those obtained on the original alcohol extracts. Since it is likely that the charcoal removed some of the sugar (3), the values reported here for sedoheptulose are minimum.

In the semiquantitative procedure aliquots of the alcohol extract of the leaf were spotted on one-dimensional paper chromatograms, developed in butanol–propionic acid–water, sprayed with orcinol-trichloroacetic acid (TCA) reagent (6), and given a relative score by inspection. Both methods of analysis for sedoheptulose indicated the same changes.

Results

Of seven varieties of Coleus tested, all contained substantial amounts of free sedoheptulose and in each the sugar was heavily labeled with C^{14} during a 10-minute period with $C^{14}O_2$. The amount of C^{14} found in sedoheptulose in 10 minutes never exceeded that found in sucrose, but in some plants it approached it closely. There were only traces of C^{14} label in free glucose and fructose. Sedoheptulose phosphate was present in amounts detectable by the orcinol-TCA spray test on the chromatograms and was labeled with a large percentage of C^{14}, but variation in its pool size has not been studied. Three of the Coleus varieties have been used in further investigations.

Diurnal Variation in Total Sedoheptulose Content: Analyses for total sedoheptulose were run on leaves collected from similar locations on the stems every hour during a day from a uniformly grown set of plants (table I). The method of analysis determined both the free sugar and probably most of its phosphate ester. Leaves of the Coleus III variety showed a several-fold change in content of total sedoheptulose during the 24-hour periods. After the first hour of light the amount of sedoheptulose was about 1.5 mg/gm fresh weight, which increased to a maximum of 5.7 mg after 6 hours of light, and then decreased during the last part of each 15-hour light period. During the 9-hour dark period, the reservoir of sedoheptulose remained small (after the first 2 hours of dark) and ranged from 0.7 to 1.1 mg/gm fresh weight.

Table I

Diurnal Variation in Sedoheptulose Content of Leaves of Three Coleus Varieties

Hrs	Coleus III		Lincoln		Golden Bedder
	Orcinol-HCl analysis, mg/gm F.W.	Chromatographic analysis, relative	Orcinol-HCl analysis, mg/gm F.W.	Chromatographic analysis, relative	Orcinol-HCl analysis, mg/gm F.W.
			Light		
1	1.5	1	3.2	3	2.2
2	3.8	4	4.6	4	2.1
3	...	...	4.3	4	2.6
4	4.0	4	4.9	5	1.7
5	...	...	6.0	5	1.2
6	5.7	4	5.1	5	1.3
7	...	...	4.2	4	1.6
8	3.0	4	2.5	3	1.4
9	...	...	3.9	3	1.8
10	0.7	tr	4.1	3	0.9
11	...	...	3.9	3	1.7
12	4.0	2	5.6	4	1.1
13	...	...	2.9	3	1.6
14	1.4	1	2.9	3	5.0
15	1.1	1	3.9	4	2.1
			Dark *		
1	1.8	2	1.8	1	1.1
2	1.2	1	...	...	4.6
3	...	...	2.3	1	2.3
4	0.7	tr	5.2	3	1.4
5	...	...	1.8	1	0.9
6	0.9	tr	4.3	3	1.2
7	...	tr	2.4	2	1.1
8	1.0	1	2.2	2	2.2
9	1.1	1	...	...	1.1

F.W. = fresh wt; tr = trace.

* Hours of darkness immediately after the 15-hr light period.

Less consistent results are reported in table I for two other varieties of Coleus. The Lincoln variety contained 2.5 to 6.0 mg of sedoheptulose/gm fresh weight, and the Golden Bedder contained 1.1 to 5.0 mg/gm leaf tissue. Neither of these varieties of Coleus showed so marked a diurnal variation in the total amount of the sedoheptulose reservoir as the Coleus III variety.

Diurnal Variation in C^{14} Labeling of Sedoheptulose: The percentage distribution of total $C^{14}O_2$ fixed into the products that were soluble in both hot alcohol and water was determined by chromatographic analyses. At each hour during the day a leaf was transferred to the photosynthetic chamber for an additional 10 minutes of light and simultaneous exposure to $C^{14}O_2$ before being killed. The distribution of C^{14} into all the soluble compounds was determined for calculations of the percentage in sedoheptulose; the percentage of C^{14} in sucrose is tabulated for comparison (table II). Total C^{14} fixed into the soluble portion amounted to about 2 to 4 % of the total $C^{14}O_2$ in the photosynthetic chamber. Total $C^{14}O_2$ fixation varied depending upon the size of the leaf. The younger smaller leaves fixed less total C^{14} per leaf, but more total C^{14} per unit weight of leaf.

The percentage of total fixed C^{14} that was incorporated into sedoheptulose during the first part of the day was higher than that during the last few hours of the light period. The time of highest rate of C^{14} incorporation into sedoheptulose corresponded with the time of maximum total sedoheptulose content shown in table I. After 6 hours in the light, the Lincoln Coleus was fixing more $C^{14}O_2$ into sedoheptulose in 10 minutes than into sucrose, yet at the beginning and end of the 15-hour day the amount being fixed in sucrose was many times as great as in sedoheptulose. In this variety the rate of formation of sedoheptulose was greatest during the middle of the day, whereas the incorporation of C^{14} into sucrose was maximum at periods of lowest sedoheptulose synthesis. In Golden Bedder Coleus, less $C^{14}O_2$ was fixed into sucrose during the day and the distribution was less consistent, probably because of substantial labeling of polysaccharides and heavy labeling of glycine and serine (30 to 50 % of the total $C^{14}O_2$ fixed). C^{14} was incorporated into sedoheptulose most rapidly during the first part of the day.

The products formed during each of the test periods by leaves harvested after increasing hours of darkness demonstrate the ability of a Coleus leaf to synthesize sedoheptulose when exposed to light. For both varieties, this rate dropped after the first 2 hours

TABLE II

DIURNAL VARIATION IN PERCENTAGE DISTRIBUTION OF TOTAL SOLUBLE C^{14} FIXED DURING 10-MINUTE $C^{14}O_2$ PHOTOSYNTHESIS

HRS	GOLDEN BEDDER					COLEUS III		
	LEAF WT, GMS	TOTAL SOLUBLE C^{14} FIXED, CPS/LEAF	DISTRIBUTION OF C^{14} *			DISTRIBUTION OF C^{14} *		
			SUCROSE, %	SEDOHEPTULOSE, %	SEDOHEPTULOSE PHOSPHATE, %	SUCROSE, %	SEDOHEPTULOSE, %	SEDOHEPTULOSE PHOSPHATE, %
				Light				
1	1.12	33,600	9.5	3.9	4.5	12.2	7.5	3.5
2	0.62	29,700	8.9	3.9	5.6	12.2	5.0	9.2
3	1.63	33,500	10.8	3.8	4.7	...	...	...
4	0.80	37,800	7.3	3.7	5.7	11.5	8.2	6.3
5	1.11	39,800	7.2	2.7	3.9	...	...	...
6	0.57	34,800	5.2	0.9	4.4	11.6	14.5	9.5
7	1.28	39,800	6.0	1.8	3.4	...	...	...
8	0.53	28,100	5.3	1.5	3.8	13.8	3.8	20.6
9	1.03	40,800	13.2	2.9	3.0	...	...	...
10	0.66	34,100	6.0	trace	3.8	16.3	8.0	5.7
11	1.03	39,500	14.4	1.2	4.6	...	...	...
12	0.48	27,400	5.3	>0.5	4.6	14.2	9.6	5.3
13	1.29	40,800	6.6	0.5	3.0	...	...	...
14	0.76	32,300	9.3	2.0	7.5	16.2	1.6	10.1
15	0.89	34,600	8.2	2.0	...	13.6	2.4	4.8
				Dark **				
1	0.60	22,400	4.9	~ 0.3	4.5	10.6	2.5	6.0
2	0.69	21,000	3.9	~ 1.7	5.3	17.8	1.6	12.0
3	0.53	15,800	8.4	~ 0.8	6.8	...	...	...
4	1.02	30,300	7.6	~ 0.6	5.5	20.4	1.2	7.1
5	0.46	23,400	6.8	trace	5.9	...	...	...
6	1.11	33,300	11.9	~ 0.4	4.7	25.1	1.4	8.7
7	0.53	20,400	7.3	trace	5.3	...	...	...
8	0.81	24,800	10.8	trace	5.3	16.7	1.1	8.2
9	0.56	21,900	10.9	trace	4.4	17.5	1.2	5.3

* Percentage of the total $C^{14}O_2$ fixed which was found in these products.

** The dark period immediately followed the 15 hrs of light in one continuous experiment. The leaf removed for analysis was given 5 min of light before the 10-min period of exposure to $C^{14}O_2$.

of darkness to a consistent low value for the rest of the night. The Golden Bedder Coleus synthesized only a trace of sedoheptulose during the first 15 minutes of light after a dark period.

C^{14} LABELING OF SEDOHEPTULOSE PHOSPHATE: The total organic phosphate ester area of each chromatogram was eluted and hydrolyzed with Polidase S. Rechromatography of the organic moieties containing the C^{14} provided identification of the original phosphate esters. Table II also shows C^{14} incorporated into sedoheptulose phosphate as a percent of the total radioactivity in the soluble extract of the leaves after a 10-minute photosynthetic period with $C^{14}O_2$. The amount of C^{14} incorporated into sedoheptulose phosphate remained large throughout a 24-hour period and no diurnal variation was found for its rate of labeling. With the Golden Bedder variety the amount of C^{14} incorporation into sedoheptulose phosphate in 10 minutes exceeded that incorporated into the free sugar at any time during the day. With the Coleus III variety incorporation of C^{14} into the phosphate ester was greater except during the middle of the day. At night, when free sedoheptulose labeling decreased to a trace, the labeling of the phosphate ester did not decrease. The total amount of sedoheptulose phosphate was not measured, but inspection of the chromatograms which were sprayed with orcinol-TCA indicated that its reservoir did not become significantly large in comparison with the free sedoheptulose pool.

EFFECT OF LIGHT INTENSITY AND AGE OF LEAF: The sedoheptulose content of leaves from about the same location on the plants often varied several-fold. Part of this variation may be explained by the light intensity, as indicated in the data of table III, and lines 2 and 3 of table IV. The coloration of the Golden Bedder Coleus leaves is dependent on light intensity, being green in the shade, yellow in bright light

TABLE III

EFFECT OF LIGHT INTENSITY ON SEDOHEPTULOSE CONTENT OF GOLDEN BEDDER COLEUS LEAVES

APPROX LIGHT INTENSITY, FT-C	LEAF COLOR	AMT OF SEDOHEPTULOSE, MG/GM FRESH WT *
300–700	Green	1.1
3000–5000	Yellow	0.6
Full sunlight	Yellow-white	0.3

* Of leaves harvested early in the morning from comparable position on plant.

TABLE IV

EFFECT OF LIGHT INTENSITY AND AGE ON SEDOHEPTULOSE CONTENT OF COLEUS LEAVES

LEAF	APPROX LIGHT INTENSITY, FT-C	SEDOHEPTULOSE, MG	SEDOHEPTULOSE, MG/GM FRESH WT
Young, uppermost	2500	1.5	4.2
Mature, middle	2000	4.8	4.8
Mature, middle	500 (shaded)	13.3	5.5
Old, lower	1500	7.9	4.0
Old, lower, dead	....	1.0	0.4

(3000 to 5000 ft-c), and a bleached yellow-white in full sunlight. The amount of sedoheptulose in these leaves decreased with increasing light intensity.

With Lincoln Coleus plants (table IV), the sedoheptulose content (fresh weight basis) was also somewhat higher in leaves illuminated at lower light intensity as well as in mature leaves. The increase in sedoheptulose content thus occurred under conditions where the photosynthesis rate was probably less. The amount of sedoheptulose per leaf was very large in the older and mature leaves of Coleus that had grown to maximum size. In old lower leaves or in abscised leaves shortly after dropping, substantial amounts of sedoheptulose were still present.

DISCUSSION

Relatively large amounts of free sedoheptulose are present in Crassulacean plants. In addition Nordall and Öiseth (10, 11) found sedoheptulose in Primulaceae and Saxifragaceae. The discovery that sedoheptulose phosphate was an important intermediate in the photosynthetic carbon cycle (2) indicated that this sugar phosphate is present in all photosynthetic tissues. This report shows that free sedoheptulose is present in as large amounts in Coleus as in the Crassulacean plants. Significant amounts of the free sugar have not however been found on chromatograms of extracts from most of the plants (barley, wheat, corn, beet) used routinely in this laboratory for metabolic studies, though sedoheptulose phosphate is detectable in all these plants by the C^{14} tracer. In a survey of many plant extracts after fermentation by bakers yeast, unidentified ketoheptoses in undetermined amounts have been detected by paper chromatography (13). Another survey[4] now in progress for sedoheptulose in plant extracts is showing that several plant genera produce large amounts of free sedoheptulose.

A function for the large reservoir of sedoheptulose in Sedum plants has not been elucidated. The size of the reservoir appeared to be very constant and did not show a diurnal variation (7, 12). In the Sedum, however, during the day the large amount of newly formed sedoheptulose from photosynthesis was continually being added to this reservoir and the sugar must have been converted to other products at a rate comparable to its synthesis (12). In the Coleus plants, a diurnal variation for both total quantity of sugar and rate of its synthesis from $C^{14}O_2$ was observed. The maxima for both synthesis and storage of sedoheptulose appeared during the day after 5 to 6 hours of light; this is similar to synthesis and storage of sucrose in other plants. Consequently free sedoheptulose would appear to be functioning as a carbohydrate reservoir.

The rate of C^{14} labeling of sedoheptulose phosphate did not vary during the day as did the labeling of the free sugar. The phosphate ester was labeled rapidly and with a large percentage of the total C^{14} fixed at all times during photosynthesis. Since the relative amount of sedoheptulose phosphate was small compared to the free sugar, it was not studied quantitatively. Thus sedoheptulose phosphate appears to serve as an active intermediate in the photosynthetic carbon cycle. In addition it is probably the precursor for the free sugar which serves as a carbohydrate reserve.

In some respects the free sedoheptulose pool in Coleus did not behave as a photosynthetic reservoir but acted in an anomalous fashion as it does in Sedum (12). In the leaves of some varieties of Coleus, the diurnal variation in amount of sedoheptulose could not be demonstrated. Shaded older leaves on the plant, which were probably less active in rate of photosynthesis and growth, contained more sedoheptulose. Furthermore, a substantial reservoir of sedoheptulose seemed to persist in the leaves. For example, even abscised leaves still contained significant amounts of sedoheptulose. These findings suggest that the free sugar may perform some as yet undetermined function, or it may be a relatively inert sugar in metabolism.

SUMMARY

One to six milligrams of sedoheptulose per gram of fresh weight of leaf was present in varieties of Coleus. The amount of sedoheptulose in the leaves increased in older leaves and decreased at higher light intensities. In certain varieties, the free sedoheptulose reservoir exhibited a distinct diurnal variation in amount and rate of synthesis from $C^{14}O_2$. These values increased during the first part of the day and decreased to a small amount at night. Rate of labeling (per 10 minutes) of sedoheptulose phosphate, on the other hand, did not fluctuate during a day and night but remained very large.

The authors wish to express their appreciation to Dr. G. E. Hunt and Dr. L. M. Rohrbaugh for their advice on this project and to Dr. L. P. Zill for aid with the methods of analysis of sedoheptulose in Coleus plants.

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PROTEIN SYNTHESIS DURING WATER UPTAKE BY TUBER TISSUE[1]

KENNETH V. THIMANN[2] AND GORDON M. LOOS[3]

Biological Laboratories, Harvard University, Cambridge, Massachusetts

The formation of protein during cell enlargement in different plant organs has been the subject of conflicting reports. In the corn (maize) coleoptile, intact and attached to the plant, Blank and Frey-Wyssling in 1941 (2) found a 160 % increase in protein nitrogen during the elongation period. Even in the filaments of rye (Secale) which elongate for only a very short time, they found (1) that an increase of 30 % in total nitrogen took place during elongation. The increase was all in the protein nitrogen fraction. The first internodes of bean plants, treated locally with auxin and growing into tumors over a 12-day period, contained 10 times as much total nitrogen as controls (17) and some of this was "water-insoluble." However, in all these attached objects the inflow of nitrogen from the seed or stalk obscures the changes occurring in the organ itself, and it is impossible to distinguish between the accumulation of nitrogenous compounds, part of which may be converted to protein, and the direct synthesis of protein from amino acids already present.

Nevertheless, even in the simpler case of isolated growing pieces of plant tissue the situation is by no means clear. Isolated segments of Avena coleoptiles, growing in auxin and sucrose solution, have in several of our experiments been examined for net protein synthesis but they have shown no change, or a slight decrease, rather than a net gain. Bonner (3) had earlier noted that auxin treatment did not increase their final protein content over that of controls grown in sucrose alone; however, since the initial values were not presented it remains unclear whether the protein content of both groups had increased or not during growth. Similarly Boroughs and Bonner (5) found that when corn coleoptile sections were stimulated to grow by auxin for 5 to 6 hours, there was no increased incorporation of labeled amino acid into the protein. They did show, however, that both auxin-treated and control sections incorporate labeled amino acids to some extent into their protein during the growth. This suggested protein breakdown and resynthesis. Isolated disks of tobacco leaves, growing in a simple mineral solution, also showed rapid incorporation of labeled amino acids, and this was promoted by light (20). It was also promoted by oxygen, though not by ATP nor by any other energy donor or substrate tested. Protein is synthesized also in growing tissue cultures, and in quantity (see 23), but the growth which is taking place there involves extensive cell division as well as cell enlargement.

Roots whose elongation was increased by auxin antagonists have been analyzed in an attempt to show that protein synthesis need not accompany elon-

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[3] Present address: American Board of Surgery, Inc., 225 South 15th Street, Philadelphia, Pennsylvania.

gation (7). It was shown that *p*-chlorophenoxyisobutyric acid nearly doubled the root length while the total nitrogen or protein nitrogen increased only a few percent. The data reveal, however, that the great increase in length must have taken place at the expense of width, for the increase in fresh weight is far less than that in length. For example, increases in root length of 88, 89 and 87 % were accompanied by increases in fresh weight of only 31, 24 and 12.4 %, respectively. To take the middle case, the 24 % fresh weight increase was associated with an 18 % increase in total N and a 30 % increase in protein N. The figures vary from one experiment to another but apparently the protein formation, though quantitatively less, is not far from proportional to the actual volume increase. It seems clear that elongation alone, without volume measurements, is an unreliable criterion of cell enlargement in roots, and on the whole the data suggest (contrary to Burström's conclusion) that some protein synthesis does accompany increase in volume.

In sections of etiolated Pisum stems studied in this laboratory there was found at first a clear decrease of amino acids during growth and a synthesis of both asparagine and protein (8), though these syntheses were only slightly promoted by optimal auxin treatment. However, in later (unpublished) experiments we have not been able to find any appreciable net synthesis of protein in these sections, in spite of the facts that elongation was just as great, and that asparagine was actively synthesized as before. Such differing results in the same tissue at different periods suggest that some proteins are being formed and others broken down so that the net balance may vary widely with small changes in the external conditions. A similar conclusion could be drawn from Boroughs and Bonner's evidence that protein turnover occurs in the coleoptile without any net synthesis.

The extent to which protein is synthesized in isolated growing tissues is important in relation to the question of whether cell enlargement necessitates a net increase in cytoplasm (see discussion in (9) and references there cited), and also to the question of whether special enzymes must be formed for growth. In any event critical examination of another object appeared desirable. Protein synthesis by cell-free particulate systems, while of great interest in other connections (20, 25) is not appropriate here because it is not easily correlated with growth. On the whole, slices of tuber tissue seemed best adapted for this purpose, since they take up water (or "grow") over a period of several days and they show marked cell enlargement in response to added auxin.

Various aspects of the behavior of isolated potato disks have been explored, including their uptake of mineral salts, of water and of oxygen. The time course of water uptake, the action of auxin and of inhibitors have been reported in detail (6, 13, 15, 18) and the characteristic changes of respiration have also been studied (12, 15, 24). Similar studies have been made on artichoke tuber disks, (4, 14). Much less study has been made of the biochemical changes accompanying these processes. As early as 1940 Steward, Stout and Preston (21, 22), showed that protein was increased, and starch converted to sugar, in potato disks during 70 hours in water at 23° C, and this was accentuated in KCl solution. During this time the disks were aerated, but auxin was of course not added, and as a result the water uptake averaged only 7 to 8 % in 70 hours, as compared with values of about 50 % in our experiments with optimal auxin concentration. Later, Reinders (18) observed a marked decrease in starch during the uptake of water, and noted that it seemed to be accelerated by auxin treatment. However, this process is not merely starch hydrolysis, since the total osmotic pressure of the tissue contents does not increase but actually decreases, and the decrease is proportional to the water uptake (10). In the work reported here (cf also preliminary report, AAAS Meeting, Boston, 1953), the net formation of protein from amino acids has been followed during water uptake in both potato and artichoke tissues. In neither tissue does any appreciable cell division occur during the period of study.

Materials and Methods

Potatoes (*Solanum tuberosum* L. var. Katahdin) were received regularly from Dr. Cunningham of the Maine Agricultural Experiment Station, stored at about 10° C and cut into disks 1 cm in diameter and 1 mm thick as previously described. The disks were well washed in running water and then soaked overnight in shallow layers of distilled water so as to reach osmotic equilibrium. Approximately 24 hours after cutting they were blotted and weighed in groups of ten, and then supported on gauze at the surface of the growth solution (13), either distilled water or naphthalene acetic acid 10 mg per liter ($= 5.4 \times 10^{-5}$ M) being used. The washed disks were allowed to continue the uptake of water for up to 8 days. This process, which is oxygen-dependent, promoted by auxin and mediated by cytochrome oxidase, will be referred to as metabolic water uptake. After 4 or 6 days the disks were again blotted, weighed and then extracted as below.

Jerusalem artichokes (*Helianthus tuberosus* L.) were obtained locally and the sections prepared just as for potatoes. After some days in auxin the artichoke disks begin to curl upwards and sometimes to form characteristic small protuberances which are due to extensive cell division; the experiments were therefore always terminated after 6 days.

Protein was determined by grinding the sections in trichloracetic acid solution with washed quartz sand. The nitrogen in the precipitate and solution were determined by micro-Kjeldahl, using selenium (Hengar boiling stone) as catalyst, with distillation into boric acid and titration with 0.02 *N* H_2SO_4. Duplicates generally agreed within 1.5 % where about 1 mg of nitrogen was being determined.

Conditions for Protein Precipitation: In the past, "insoluble nitrogen" or "protein nitrogen" has

TABLE I

PRECIPITATION OF NITROGEN BY TRICHLOROACETIC ACID (TCA) IN WATER EXTRACT OF POTATO DISKS BEFORE AND AFTER GROWTH

STATE OF SECTIONS		% CONC OF TCA			
		2.5	5	10	20
		mg N/gm initial fresh wt			
Initial	N in TCA ppt	0.41	0.48	0.51 *	...
	N in filtrate	1.83	1.80	1.80 *	...
	Heat coagulable N in filtrate	0.15	0.21	0.10 *	...
	= % of filtrate N	7.9 %	11.3 %	5.5 %	...
After 4 days growth in NA	N in TCA ppt	1.21	1.30	1.39	1.34
	N in filtrate	0.97	0.83	0.81	0.83
	Heat coagulable N in filtrate	0.08	0.00	0.00	0.00
	= % of filtrate N	8.2 %	0 %	0 %	0 %

* Mean of two separate experiments.

been determined by precipitation with alcohol or with trichloracetic acid of various strengths and under varying conditions and temperatures. Particularly with trichloracetic acid the concentrations used in different laboratories have varied from 2.5 to 10 % and the temperature from zero to boiling. Steward and Preston (21) compared cold 2.5 % trichloracetic acid with alcohol and found that the former gave lower values, but as far as synthesis of protein was concerned, i.e. difference between initial and final values, the two methods agreed. The trichloracetic method has been discussed by Kirk (16; cf also the modification used by Siekewitz, 19). Since it was anticipated that details of the method would prove critical, a preliminary study was made of the influence of several concentrations of TCA on the protein determination in potato slices.

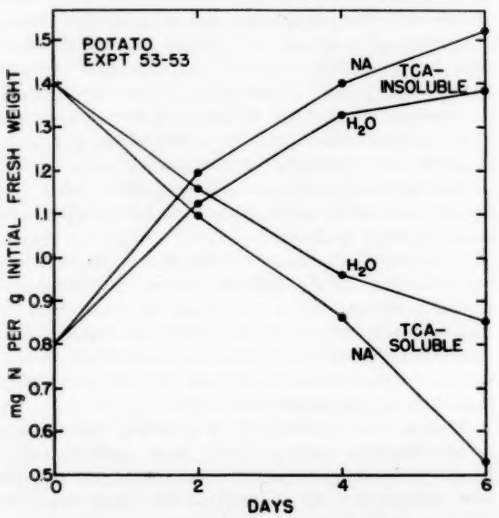

FIG. 1. Increase in protein nitrogen and decrease in soluble nitrogen in a representative experiment with potato disks. Note that at zero time the disks have already been equilibrated in water for 24 hours. NA = naphthalene-acetic acid, 10 mg/liter, pH 5.5.

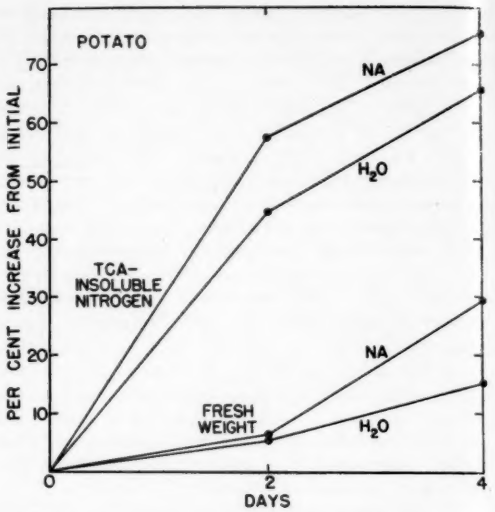

FIG. 2. Average increase in protein nitrogen in 5 series, compared with the average uptake of water by the same disks. Data reduced to % increase over initial values.

The tissue was ground in the coldroom at 3° C. The filtrate was divided into 2 aliquots, one being subjected to the Kjeldahl directly, the other being boiled for 3 minutes, filtered, and the nitrogen of filtrate and precipitate determined. Table I shows results with 3 concentrations of trichloracetic acid (TCA) and two types of tissue, namely slices cut and equilibrated in the usual way in distilled water for 24 hours ("initial"), and slices similarly equilibrated and then allowed to take up water for 4 days in presence of naphthalene acetic acid (10 mg per liter).

It will be seen that the nitrogen in the TCA ppt increases significantly up to 5 or 10 % TCA. With the slices equilibrated only for 24 hours, some heat-

TABLE II

TCA-INSOLUBLE NITROGEN OF POTATO SLICES

EXPT NO.	MG N/GM INITIAL FRESH WT				
	INITIAL	AFTER 4 DAYS' GROWTH			
		IN H_2O	INCREASE	IN NA	INCREASE
1	0.95	1.46	0.51	1.65	0.70
2	0.80	1.33	0.53	1.40	0.60
3	0.88	1.46	0.58	1.51	0.63
4	0.86	1.40	0.54	1.45	0.59
5	0.76	1.38	0.62	1.50	0.74
Av	0.85	1.41	0.56	1.50	0.65

TABLE III

TCA-INSOLUBLE AND SOLUBLE NITROGEN OF ARTICHOKE SLICES BEFORE AND AFTER WATER UPTAKE

DAYS	MG N/GM INITIAL FRESH WT *			
	INSOLUBLE	SOLUBLE	SUM	TOTAL FOUND DIRECTLY
	Initial **			
0	1.18	2.08	3.26	3.28
	Disks in water			
2	1.36	1.90	3.26	...
4	1.23	2.10	3.33	...
6	1.16	2.04	3.20	...
	Disks in NA			
2	1.83	1.34	3.17	...
4	1.72	1.70	3.42	...
6	1.59	1.70	3.29	...

* Determined by grinding 15 min with 10 % TCA.
** The initial sample was the same for both water and NA treatments.

coagulable nitrogen always escapes precipitation, though it makes up only a small fraction of the nitrogen in the filtrate. With the 4-day material, however, 5 % TCA appears to precipitate all the heat-coagulable nitrogen. It seems, therefore, that, particularly in the "initial" slices, there is no sharp line between the TCA-soluble and insoluble fractions, and that substances resembling protein at least in their heat-coagulability may escape precipitation even with 10 % TCA. These borderline materials evidently disappear during the four days of water uptake. It is suggested that they consist of polypeptides of moderate size (cf 16).

As a result of these experiments the use of 10 % TCA in the cold was standardized for all subsequent determinations. It is to be understood that the term "protein nitrogen" as used below refers to the nitrogen fraction insoluble in 10 % TCA in the cold.

PROTEIN SYNTHESIS IN POTATO DISKS: Protein and non-protein nitrogen were determined in this way on potato disks equilibrated in water for 24 hours and after a subsequent 2, 4 and 6 days of metabolic water uptake. The results of a typical series are plotted in figure 1. It will be seen that at the start the amino acids and other soluble compounds compose 63 % of the total nitrogen, but that they decrease, and the protein nitrogen increases, throughout the 6-day period (cf the data of Steward et al 21, 22). The change is intensified by the naphthaleneacetic acid (NA) but also occurs in water alone. In the last 2 days the decrease in soluble nitrogen is a little greater than the increase in protein, suggesting that the tissues are losing nitrogen to the external solution. In this experiment the total loss by the 6th day amounted to 6.3 % of the initial nitrogen.

The amount of protein synthesized by the disks is very reproducible from one experiment to another. Table II summarizes the initial and 4-day values of five separate experiments and shows that the increase of protein, in the disks in water, averages 0.56 mg, or some 66 % of the initial protein value. The data also show that the effect of auxin, though small, is definite. It is clearly present, and of the same magnitude, in each one of the five series. The average increase in all the experiments with NA is 0.65 mg, or 77 %. Thus auxin promotes the protein synthesis by 10 to 20 %.

It should perhaps be noted that a limited amount of protein synthesis can be brought about in potato disks by neutral salts alone (21, 22). Steward et al (22) found that 0.05 M KBr increased the protein synthesis, during 70 hours, by 2.4 %. But since the amount of K^+ added to the NA solution for neutralizing was in the present experiments less than $5 \cdot 10^{-5}$ M it is evident that the effect here described is genuinely due to the auxin.

Figure 2 shows that the larger part of the protein synthesis, and all of the increase due to NA, takes place within the first 2 days. These values are the means of all 5 series. During this two-day period of active protein formation the water uptake is relatively small and is barely affected by the auxin. In the latter respect the data agree exactly with those

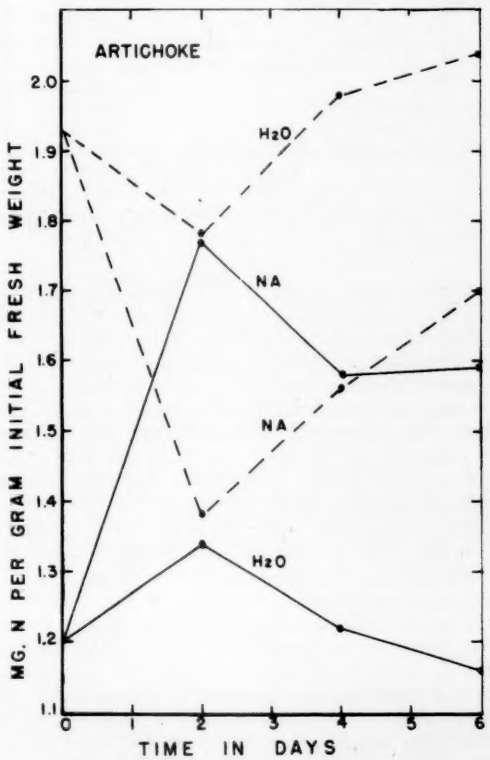

FIG. 3. Increase in protein nitrogen (solid line) and changes in soluble nitrogen (dashed lines) in artichoke tuber disks. Mean of two similar experiments for 2 and 4 days, single experiment for the 6th day.

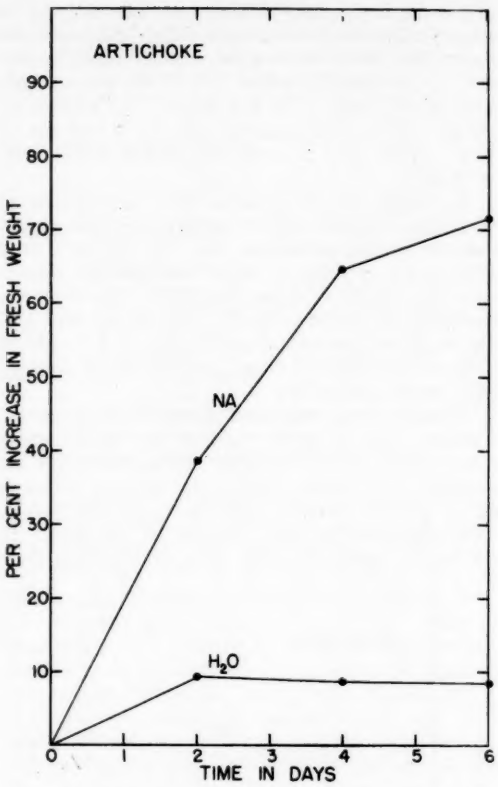

FIG. 4. Uptake of water by the same artichoke disks as in figure 3.

obtained earlier (cf 13, fig 2), though the effect of the NA is very slightly smaller than previously found (30 % increase in fresh weight in 4 days against 36 % found in 1952). Thus the action of auxin on protein synthesis precedes its effect on water uptake.

PROTEIN SYNTHESIS IN ARTICHOKE DISKS: The data of an experiment with artichoke disks is shown in table III and the mean values of two similar experiments are plotted in figure 3. Again the protein nitrogen increases rapidly at first and the non-protein nitrogen decreases. This material, however, differs from the potato in that the initial synthesis of protein reverses after two days and the soluble nitrogen begins to increase again. At this time the disks were still healthy and, at least in NA, taking up water rapidly; water uptake continues steadily through the 4th day as shown in figure 4 (cf 14, fig 1). The rate of uptake shows no marked increase after two days as with potato. The totals included in table III show that there was no appreciable loss of nitrogen to the solution at any time.

Another difference from potato is that the effect of naphthaleneacetic acid is relatively far greater in artichoke, the maximal amount of nitrogen converted to protein (i.e. increase over the initial value) being 0.57 mg/gm initial fresh weight in NA as against 0.14 mg/gm in water. Thus the NA increases the protein synthesis by 400 %.

DISCUSSION

The clear-cut action of naphthaleneacetic acid in promoting protein synthesis, especially in artichoke, is an unequivocal demonstration that auxin in physiological concentrations can modify large-scale or "bulk" metabolic processes. Other workers have sometimes found changes in enzyme content to follow auxin treatment, and respiration rate is frequently increased, but the respiratory substrate whose oxidation is facilitated by auxin has not been directly identified. Small changes have also been detected in the metabolism of specific cell-wall constituents, although their importance in growth is yet to be demonstrated. The question to be answered here is how far the protein synthesis is related to growth. Three points will be made.

In the first place, since (a) protein synthesis is maximal in the potato when growth has scarcely begun, and (b) synthesis gives way to protein breakdown in the artichoke after 2 days, while growth continues unabated, it follows that the synthesis cannot be related to growth in any simple way. In this respect protein synthesis in these tubers is not different from that in the elongating stem and coleoptile sections discussed in the introduction.

On the other hand the comparison between the potato and artichoke is most suggestive. In the potato, auxin increases protein synthesis to only a limited (though fully significant) extent, say 10 to 15 %, and correspondingly the increased growth over that of the controls is only 30 to 40 %. In the artichoke, auxin increases protein synthesis by 400 % and correspondingly growth is increased by almost 1000 % over that in the water controls (fig 4). The respiration was also found to be increased by 400 % (14). In both tissues the protein synthesis comes early and tapers off while growth is continuing. Evidently, therefore, if there is a connection at all, the protein synthesis is a cause rather than an effect, and its magnitude is roughly commensurate with the growth-effect. Since the absolute protein snythesis, and also the growth, in the water controls is much less in the artichoke than in the potato, it may be suggested that the absolute auxin level in this control tissue is correspondingly lower than in the potato.

Finally, potato slices have the curious property of changing their terminal oxidase after cutting, as well as increasing its apparent quantity (24, and earlier work there cited). If this involves the breakdown of part of the cytochrome system and its resynthesis in modified form, then the observed protein synthesis may be in part a synthesis of this and perhaps other enzymes. Hackett's recent observation (11) that the change in the terminal oxidase does not occur in nitrogen or in cyanide is in line with this view. That a qualitative change in the potato proteins does take place is furthermore indicated in table I, where the

heat-coagulable material soluble in TCA is seen to disappear after 4 days. Such a partial breakdown and resynthesis of proteins, including enzymes, in cut sections would easily explain the lack of overall synthesis in some tissues and its presence in others, as well as the evidence of protein turnover without net synthesis (5). It would explain, too, the reported increases of several enzymes in tissues which have been exposed to auxin. Thus the promotion of protein synthesis by auxin, even if it applies only to a part of the total proteins of growing cells, may have profound effects on the enzymatic content, and therefore the metabolism, of the tissues.

Summary

Synthesis of protein (trichloracetic acid insoluble nitrogen) and metabolic uptake of water have been followed simultaneously in slices of potato and artichoke tuber.

In freshly cut potato slices a small amount of heat-coagulable nitrogen escapes precipitation with TCA. This fraction, which may consist of polypeptides, disappears after 2 to 4 days of water uptake.

Protein is rapidly synthesized in potato slices during the first two days after equilibration, when water uptake is small. It tapers off in the next 2 to 4 days while water uptake greatly increases.

Artichoke shows similar behavior, with the difference that during the second 2-day period synthesis gives way to a net hydrolysis.

In both tissues naphthalene-acetic acid markedly increases protein synthesis in the first two days. The magnitude of the increase is roughly commensurate with that of the increase in water uptake.

It is suggested that the proteins synthesized include enzymes, and that this phenomenon may explain the frequently observed increase in enzyme activity following auxin treatment.

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MOLYBDENUM AS A PLANT NUTRIENT. X. SOME FACTORS AFFECTING THE ACTIVITY OF NITRATE REDUCTASE IN CAULIFLOWER PLANTS GROWN WITH DIFFERENT NITROGEN SOURCES AND MOLYBDENUM LEVELS IN SAND CULTURE[1]

M. I. CANDELA,[2] E. G. FISHER[3] AND E. J. HEWITT

AGRICULTURAL RESEARCH COUNCIL UNIT OF PLANT NUTRITION (MICRONUTRIENTS), LONG ASHTON RESEARCH STATION, UNIVERSITY OF BRISTOL, ENGLAND

Nitrate reductase enzymes were obtained in partially purified states from *Neurospora crassa* and soybean by Nason and Evans (16) and Evans and Nason (8) who showed also that the enzyme is widely distributed with greatly differing activity in several higher plants. The presence and role of molybdenum as a specific component of these enzymes was shown by Nicholas and Nason (17, 18, 19).

Evidence has been presented elsewhere by Agarwala (1), Agarwala and Hewitt (3), Hewitt and McCready (13) and Nicholas, Nason and McElroy (19) that molybdenum is also required by plants and fungi when provided with nitrite, ammonium compounds, urea, or other sources of nitrogen and that the role of molybdenum in nitrate reductase may not entirely explain its function as an essential element. This conclusion, together with the evidence of Evans and Nason (8), who reported that they obtained no responses to different nitrogen sources or molybdenum supplies in regard to their effects on the activity of nitrate reductase, suggested that further investigation of some of the factors affecting the activity of the enzyme was desirable.

The investigations reported here were made on cauliflower, as this plant has been extensively used at Long Ashton in previous studies concerning molybdenum, with special reference to the origin of the disorder known as whiptail (2, 3, 10, 11).

MATERIALS AND METHODS

Cauliflower plants (*Brassica oleracea* L. var. *botrytis* cv. Majestic) were grown in sand culture using methods already fully described (3, 9). Nitrogen was given as nitrate or as nitrite or ammonium sulphate usually at 12 millimoles/liter. Molybdenum was given at 0.05 ppm, 10 to 20 ppm or was omitted. The residual level in the molybdenum omitted treatments was probably the equivalent of 0.00001 to 0.00002 ppm. Plants grown at this level developed typical severe symptoms of whiptail already described (2, 3). The symptoms were produced in all three nitrogen treatments but plants grown with nitrate showed also the characteristic mottling and necrosis that occur under these conditions (3, 13).

ENZYME EXTRACTION AND SAMPLING: Nitrate reductase was extracted by grinding fresh tissue for 15 minutes with neutral acid-washed silica sand and three times its weight of extractant at 0° C in a chilled mortar. The extracting reagent in 1954 was 0.1 M phosphate buffer pH 7.0 containing 10^{-4} M cysteine and ethylenediamine tetraacetate (EDTA) (18). In 1955 the buffer pH was 8.8. The ground tissues were filtered through muslin and the extract was centrifuged at 20,000 × g at 0° C and kept in ice or used immediately. Activity observed in these extracts was also compared with that obtained by grinding at 0° C in mechanically driven close-fitting glass macerators, by disintegration in a high speed blendor, or by macerating in a hand operated Ten-Broeck pattern glass macerator.

In 1954 the leaf lamina of entire plants was used except that the two oldest or other senescent leaves and those under about 2 cm in length were discarded. In 1955 plants were subdivided into pairs of leaves from adjacent nodes and into root, stem and petiole which were examined separately. It was not possible to sample at one time from all treatments. Simultaneous comparisons were made in regard to molybdenum status whilst the nitrogen treatments were sampled consecutively on three or more occasions in random order. In some tests the plant material was stored at −18° C before assay but the same storage treatment was used in all comparative tests. There did not appear to be any loss of nitrate reductase over 24 hours under these conditions.

NITRATE REDUCTASE ASSAY AND PREPARATION OF REAGENTS: Two tenths or 0.5 ml of extract were used almost immediately in the following reaction system: 1.0 ml 5 % sodium nitrate; 0.1 ml reduced diphosphopyridine nucleotide (DPNH 1 mg/ml); 0.05 ml boiled extract of pig heart acetone powder; 2.5 or 2.8 ml of 0.2 M phosphate buffer pH 7.3; distilled water to produce 5.15 ml. The assays were carried out at 27° C for 20 minutes in 1954 or for 10 minutes at 28° C in 1955. The respective rates of reaction were constant over these intervals. Nitrite was estimated as described by Evans and Nason (8) with the addition of sufficient hydrochloric acid to obtain maximum colour development. Blank estimations were carried out by omitting DPNH and all assays were made in duplicate. Activities were expressed as total activity: millimicromoles of nitrite produced in 10 or 20 minutes per 0.2 or 0.5 ml of extract and as specific activity: millimicromoles nitrite in one minute per mg

[1] Received December 18, 1956.

[2] Present address: Instituto de Edafologia y Fisiologia Vegetal, Serrano, 113, Madrid, Spain.

[3] Present address: New York State College of Agriculture, Agricultural Experiment Station, Cornell University, Ithaca, New York.

TABLE I

EFFECT OF METHOD OF GRINDING ON NITRATE REDUCTASE ACTIVITY IN CAULIFLOWER LEAF EXTRACTS *

EXPT	METHOD OF GRINDING	RATIO OF BUFFER TO LEAF WT		
		1/3	1/4	1/5
1	Mortar	154	...	200
	N.I.R.D. blendor	...	...	177
2	Mortar	133	...	...
	N.I.R.D. blendor	...	...	140
	Mechanical glass	91	47	...
3	Mortar	84	...	110
	Mechanical glass	26	37	20
4	Mortar	96	97	102
	Ten-Broeck	59	...	80
5	Mortar	137	...	...
	Mechanical glass	73	...	...
6	Mortar	179	...	...
	Mechanical glass alone	133	...	136
	With ground glass	79	...	58

* Millimicromoles nitrite produced in 10 min per gm leaf wt.

protein. Protein was estimated colorimetrically by the biuret reaction (21) or turbidimetrically in 1954, and turbidimetrically after precipitation with 2 % trichloracetic acid in 0.06 M ammonium sulphate in 1955.

The presence of nitrite in solutions of analytical reagent grade sodium nitrate and other reagents was minimised by preparing daily a fresh solution of nitrate to which 0.5 mg ammonium chloride was added followed by boiling for three minutes, and also by selecting reagents for minimum nitrite content. The DPNH was prepared from 60 to 75 %. DPN obtained from yeast or commercially. It was reduced in 0.04 M pyrophosphate buffer containing 0.2 M ethyl alcohol by addition of crystalline alcohol dehydrogenase prepared from yeast as described by Racker (20). The pH was maintained between 9.8 and 10.0 with 2 *N* KOH during the reaction which was observed spectrophotometrically at 340 mμ. Approximately 100 mg actual DPN were used in 70 ml and about 70 to 80 % of the DPN was reduced. At the conclusion of the reaction the solution was heated for 5 minutes in a boiling water bath, cooled rapidly and stored at −18° C. The pig heart extract used to provide FAD (8) was prepared by boiling one part of an acetone powder of pig heart in three parts of water for 5 minutes, centrifuging and storing at −18° C.

ACID PHOSPHATASE ASSAY: Acid phosphatase activity was also determined in some extracts. The two enzymes showed markedly different trends in activity in regard to the regions from which they were extracted. This disposed of the possibility that large differences observed between the activity of nitrate reductase in samples from different regions might be due merely to a variable degree of disruption of the cells in different extractions. Acid phosphatase was assayed as the rate of hydrolysis of phenolphthalein phosphate at 27° C over a 15-minute period at pH 5.4 in 0.075 M acetate buffer. The reaction was stopped by adding 1 ml 15.5 % trichloracetic acid. The colour of liberated phenolphthalein was developed on addition of 1 ml *N* sodium hydroxide to bring the solution to pH 10 approximately and was measured with a Spekker absorptiometer within 10 minutes, using green filters and a calibration curve prepared from phenolphthalein.

RESULTS

EFFECT OF GRINDING PROCEDURE AND pH: Results in table I show that grinding in a mortar with sand or in the high-speed blendor gave the highest ac-

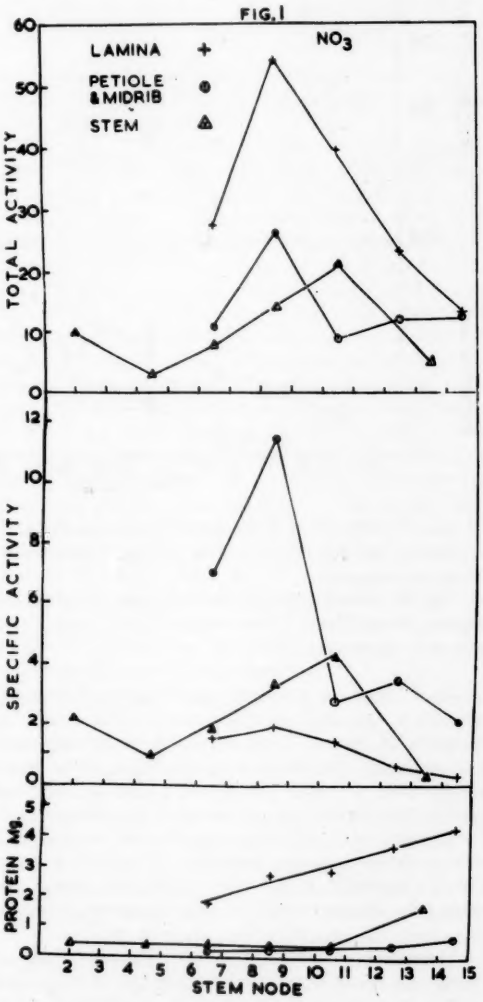
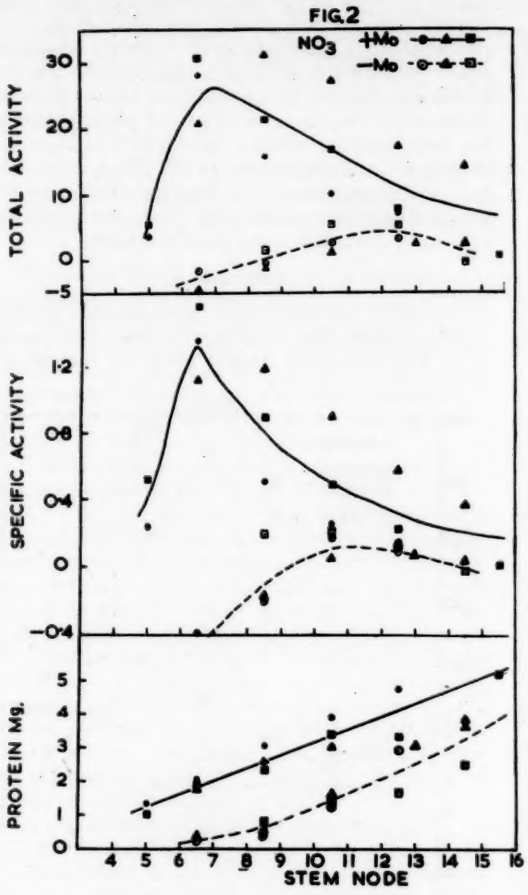


FIG. 1. Nitrate reductase in different regions of cauliflower plants grown with nitrate.



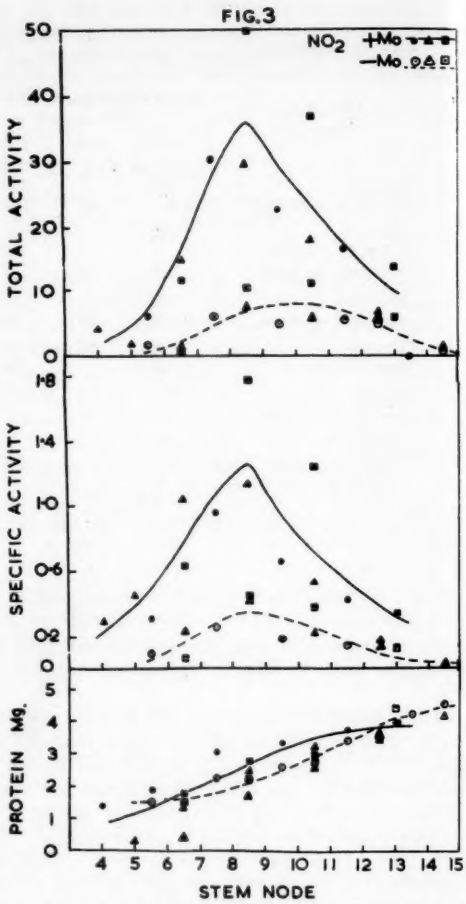


FIG. 2. Effects of leaf position and molybdenum supply on distribution of nitrate reductase activity and protein in cauliflower grown with nitrate. Mean curves for 3 separate experiments shown as squares, circles and triangles, respectively.

FIG. 3. Effects of leaf position and molybdenum supply on distribution of nitrate reductase activity and protein in cauliflower grown with nitrite. Mean curves for 3 separate experiments shown as squares, circles and triangles, respectively.

tivities. Extracts prepared with the glass macerators were, however, usually low in activity and the inclusion of ground glass appeared to be still more unfavourable. The ratio of extracting fluid to tissue weight over a range between 3/1 and 6/1 did not greatly affect the activity per unit leaf weight.

The activity of the centrifuged extract was greatest with a sharp optimum between pH values of 7 and 7.4 in phosphate buffer for cauliflower grown with nitrate in contrast with purified soya bean enzyme for which the optimum was about 6 (8).

DISTRIBUTION OF ACTIVITY AND PROTEIN: The activities observed were the net activities without correction for effects of nitrite reductase. Preliminary tests indicated that nitrite reductase activity was relatively high in roots and low in aerial parts under the conditions of assay. Inclusion of hydroxylamine hydrochloride from one half, up to double the amount used by Evans and Nason (8) to inhibit nitrite reductase did not appreciably affect the observed activity of nitrate reductase in leaf extracts. It was concluded that nitrite reductase activity of these extracts was not sufficient to interfere with the general conclusions reached in this work.

In plants given nitrate with molybdenum (figs 1, 2), the net activity was greatest in mature fully expanded, though not senescent leaves, adjacent to the oldest two or three leaves remaining on the plant at the time. Net activity decreased progressively in younger leaves. The distribution in leaves of plants grown with nitrite or ammonium sulphate and molybdenum (figs 3, 4) was similar to that in plants

given nitrate, but the decrease from the maximum was relatively less sharp in young leaves of plants given ammonium sulphate. In plants aged 16 to 20 weeks the maximum activity was usually found in leaves at nodes 6 and 7 or 8 and 9 (figs 1, 2, 3).

The distribution of net nitrate reductase activity in petioles (fig 1) resembled that in leaves with somewhat lower values. In stems the maximum occurred in younger sections than in leaves. No nitrite production could be detected using extracts of roots. Measurements on loss of nitrite in the presence and absence of added nitrate, suggested that nitrate reductase activity was low in roots.

The distribution of extractable protein differed from that of nitrate reductase and increased from oldest to youngest leaves (fig 1 to 4). The changes were marked in laminae and small in petioles. The protein extracted from stems was the same for lower and mid-stem sections and increased markedly in the youngest stem region. The distribution of specific activity resembled that of total activity, but the changes were relatively smaller between old regions and those with maximal activity and relatively greater between these and the young regions. Specific activity figures were especially high for petioles owing to the low protein values (fig 1).

Age of Plant: The net total activities in leaves of plants grown with 10 to 20 ppm molybdenum and the three sources of nitrogen in 1954 (table II) were considerably less for plants aged 20 weeks than those aged 12 weeks sampled at the same time. The specific activities, however, were similarly in both age groups for nitrite and ammonium treatments due to the lower protein extracted from the older plants, but specific activity also was decreased in the older plants grown with nitrate.

FIG.4

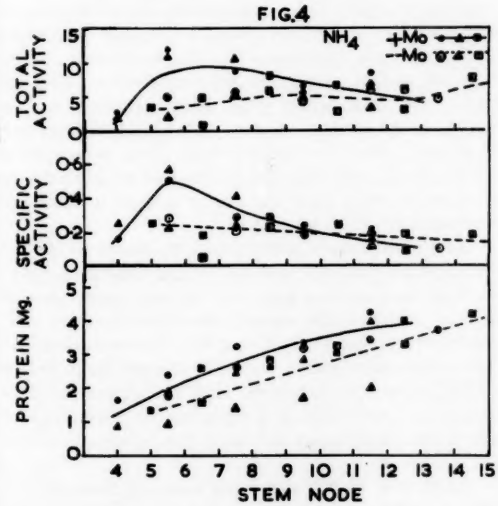


Fig. 4. Effects of leaf position and molybdenum supply on distribution of nitrate reductase activity and protein in cauliflower grown with ammonium sulphate. Mean curves for 3 separate experiments shown as squares, circles and triangles, respectively.

Table II

Effects of Nitrogen Source and Age of Plant on Nitrate Reductase Activity in Leaves of Cauliflower Grown in 1954

Nitrogen supply	Age of plants, wks	Total activity*	Protein, mg/0.5 ml	Specific activity**
Nitrate				
Normal level	12	21.6	3.2	1.68
	20	12.5	2.3	1.36
Double level	20	17.7	3.2	1.38
Nitrite	12	15.8	3.4	1.16
	20	13.5	2.8	1.20
Ammon. sulphate	12	10.4	3.0	0.87
	20	5.9	1.7	0.87

* Millimoles nitrite in 10 min per 0.2 ml extract.
** Millimoles nitrite in 1 min per mg protein.

Effects of Nitrogen Source and Molybdenum Level: The net total and specific activities (figs 1 to 4, tables II and IV) were decreased in the order nitrate > nitrite > ammonium sulphate often with significance at 0.1 % in plants aged 12 weeks given 10 ppm molybdenum in 1954. In tests with plants aged 20 weeks the total activities were similar with nitrate and nitrite and much less with ammonium sulphate. Plants aged 20 weeks given double the normal nitrate level had higher total and similar specific activities compared with those given the lower level. In 1955 the maximum values and distribution of net total activities were similar in plants given nitrate or nitrite in the presence of 0.05 ppm molybdenum. In plants given ammonium sulphate the maximum values were between one-third and one-sixth of those observed in the other two nitrogen treatments (figs 2, 3, 4). The changes in amounts of protein extracted from leaves at successive nodes were similar in the three nitrogen treatments in the presence of molybdenum and the effects of nitrogen source on relative specific activities resembled in general their effects on total activities.

In the absence of molybdenum (figs 2, 3, 4) the differences in net total activities between the different nitrogen sources were smaller. Values in leaves of plants given nitrite or ammonium sulphate, however, were perceptibly greater than those in plants given nitrate, possibly because the protein extracted from successive leaves was much less in the nitrate treatment than in the other two. For the same reason the specific activities were similar for the different nitrogen treatments in the molybdenum deficiency series.

Omission of molybdenum markedly decreased net total nitrate reductase activity in nitrate and nitrite treatments (figs 2, 3), but the effect was much smaller in plants given ammonium sulphate, where values even with molybdenum, were generally low (fig 4).

Total activities were distributed more uniformly in deficient than in normal plants, and maxima were less marked especially in plants given ammonium sulphate. In plants given nitrate without molybdenum (fig 2) activity usually increased from oldest to youngest leaves and net activities in old leaves were sometimes apparently negative. This was attributed to a slight excess of nitrite reductase activity over that of nitrate reductase in these plants. The relative effect of molybdenum status on nitrate reductase activity varied greatly and ranged from a ratio of over 15 to 1 to less than 1.5 to 1 in different regions of plants grown with or without molybdenum. The maximum values in molybdenum deficient plants tended to occur in leaves that were attached at higher nodes than those on normal plants.

Molybdenum deficiency markedly decreased the protein extracted from leaves of plants grown with nitrate (fig 2). Smaller differences were also observed in plants grown with nitrite or ammonium sulphate where values observed in leaves from any pair of nodes on molybdenum deficient plants often compared fairly closely with those in leaves from the pair of next older nodes on the normal plants (figs 3, 4). Specific activities were also generally decreased by molybdenum deficiency especially in plants grown with nitrite or ammonium sulphate, and did not show the marked maximum values at intermediate stem nodes observed in normal plants (figs 3, 4).

Effect of Leaf Infiltration by Molybdate on Nitrate Reductase and Acid Phosphatase: Leaves were removed from molybdenum deficient plants and divided into half laminae. One half was infiltrated several times whilst immersed in a 5×10^{-5} M solution of sodium molybdate by alternate evacuation and admission of air, until apparently watersoaked, and

Table III

Effects of Infiltration of Paired Half-Leaves of Molybdenum Deficient Cauliflower Plants with 10^{-5} M Molybdate on Nitrate Reductase Activity *

Source of leaf	Nitrogen treatment	Infiltration treatment: Water Range	Water Mean	Molybdate Range	Molybdate Mean
Random leaves	Nitrate	1.0–10.8	3.85	−1.4–21.4	6.60
Random leaves	Nitrite	1.2– 7.7	4.04	5.3–14.1	9.08
Leaves from successive nodes					
(i)	Nitrite	3.3– 7.2	5.14	2.6–12.8	7.28
(ii)	"	2.7–10.1	5.68	2.7– 8.9	5.90
(iii)	"	3.7– 9.1	6.40	5.6–10.8	8.67
Random leaves	Ammon. sulphate	2.0– 7.2	4.60	3.0–11.4	8.22

* Millimicromoles nitrite produced in 10 min by 0.5 ml extract.

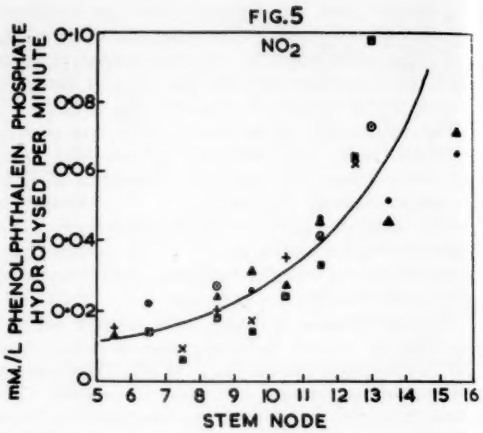


Fig. 5. Effect of leaf position on distribution of acid phosphatase activity in 8 sets of assays with cauliflower plants grown with nitrite. Paired half leaves:

▲ ■ ● ◉ Infiltrated with molybdate.
+ × ◬ ◘ Infiltrated with water.

the other half was similarly infiltrated with water as a control. The fluid absorbed was determined by the change in weight. Before extraction the leaves were kept in a saturated space for 18 to 24 hours during which they remained turgid and eliminated the surplus water. Several tests were made with single leaves chosen at random from lower nodes, and a few tests were made in which a series of leaves was sampled in relation to stem nodes. The results are summarised in table III. Infiltration by molybdenum increased the activity in most of the tests regardless of the nitrogen treatment but the magnitude of the response varied greatly in different leaves. No response to molybdenum was observed when it was added at the time of maceration. Marked increases in nitrate reductase activity have been observed in whole plants and detached leaves two to three hours after introducing molybdenum or nitrate in later experiments by E. J. Hewitt.

Acid phosphatase activity was measured in the infiltration experiments and results are shown in figure 5. The distribution was quite distinct from that of nitrate reductase and closely resembled that for protein shown in figures 2, 3, and 4. The acid phosphatase values increased in a regular manner from old to young leaves and suggested that the extraction procedure used was valid and reproducible. No effect of molybdate infiltration on acid phosphatase activity was observed.

Effect of Light and Darkness on Nitrate Reductase: These tests were prompted by two considerations, namely (i) the possibility that prolonged darkness might cause sufficient protein loss to decrease enzyme activity; and (ii) the conclusion by Burström (6) and others, that light has a specific function in nitrate reduction in some plants. Plants

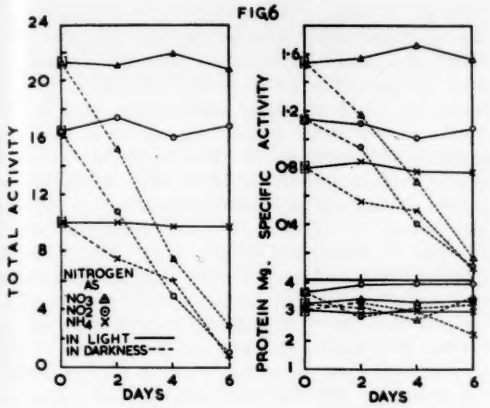


FIG. 6. Nitrate reductase activity in leaves of cauliflower grown with different sources of nitrogen in daylight or darkness.

aged 13 weeks which had been grown with nitrate, nitrite or ammonium sulphate at the high molybdenum levels in 1954 were placed in darkness in the greenhouse or were otherwise allowed to grow in the normal way. Total and specific nitrate reductase activities were determined in triplicate samples from normal and darkened plants at two-day intervals. The results are shown in figure 6. There were marked decreases in the total and specific activities significant at the 0.1 % level for plants of each nitrogen treatment whilst they were kept in darkness. Total extractable protein did not show changes of corresponding magnitude. Similar results were observed with plants aged 21 weeks in the second experiment (fig 7) which was not replicated because of shortage of material. It was found that when normal light was restored there were rapid increases in the total and specific activities in plants from each nitrogen treatment. The changes that were detectable during the first three hours of daylight continued over a period of 24 hours until the following day but the main increases occurred in the first 12 hours. There also appeared to be a net synthesis of protein during the first 6 hours of daylight, which produced slightly higher values than those observed initially.

EFFECTS OF EXCLUSION OF AIR: As the maintenance of organised metabolic activity sometimes depends on normal respiration, the effects of exclusion and reintroduction of atmospheric oxygen were tested. Plants grown with each of the nitrogen treatments were enclosed in transparent airtight plastic covers that were sealed around the outside of the pots. Nitrogen was passed rapidly through a tube into the interior to displace air and a slow stream of nitrogen was then maintained to exclude air as far as possible for 2 days. At the end of this period the plants were sampled and nitrate reductase activity was estimated. Results for plants aged 13 weeks in December, 1954 are given in table IV. In the second experiment in February 1955 with plants aged 21 weeks, the changes that occurred after the restoration of normal atmospheric conditions were also observed and are shown in figure 8. Total and specific net activity of nitrate reductase was greatly decreased by exclusion of air.

TABLE IV

EFFECTS OF 48 HOURS EXCLUSION OF AIR ON NITRATE REDUCTASE ACTIVITY IN LEAVES OF CAULIFLOWER PLANTS AGED 13 WEEKS (1954 EXPT)

NITROGEN SUPPLY	ATMOSPHERE	TOTAL ACTIVITY *	PROTEIN, MG/0.5 ML	SPECIFIC ACTIVITY **
Nitrate	Air	22.0	3.1	1.77
	N_2	11.1	3.6	0.77
Nitrite	Air	18.5	3.4	1.36
	N_2	8.0	2.8	0.71
Ammon. sulphate	Air	9.0	3.4	0.66
	N_2	8.0	3.9	0.51

* Millimoles nitrite produced in 10 min by 0.2 ml extract.

** Millimoles nitrite produced in ½ min per mg protein.

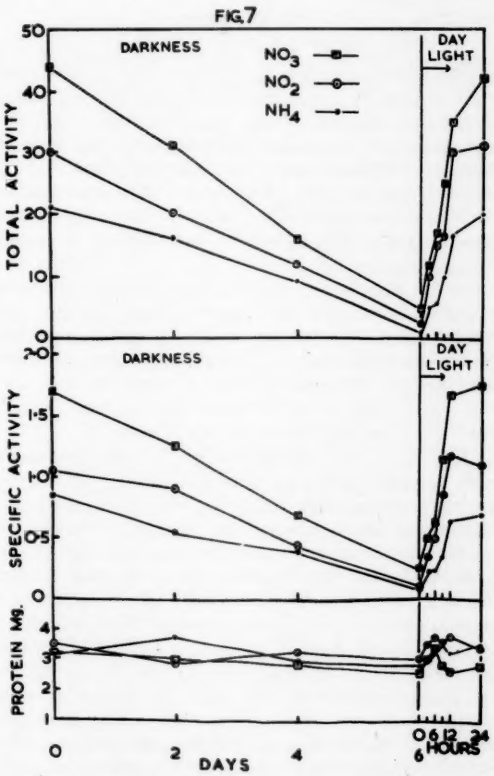


FIG. 7. Effect of darkness and restoration of light on nitrate reductase activity in leaves of cauliflower grown with different sources of nitrogen.

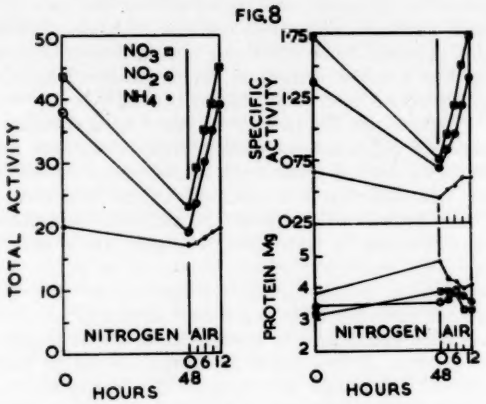


FIG. 8. Effect of exclusion and re-admission of air on nitrate reductase activity in leaves of cauliflower grown with different sources of nitrogen.

The activity was also rapidly restored on re-admitting air to the plants. No consistent trends were observed for the amounts of extractable protein.

DISCUSSION

The net nitrate reductase activity in cauliflower is determined by several factors. The pronounced peak in activity associated with mature but not senescent leaves of normal plants is noteworthy. This distribution was practically independent of the type of nitrogen nutrition and clearly distinct from that of another enzyme, acid phosphatase. The distribution of total extractable leaf protein and nitrate reductase are relatively independent at some stages, although in ageing plants total activity fell as leaf protein decreased. The total activities observed in cauliflower appeared to exceed considerably those reported by Evans and Nason (8) for soya bean and many other plants when the data are recalculated on a comparable basis. Maximal activity occurred in the primary and young trifoliate leaves of soya bean in contrast to the mature leaves of cauliflower.

The effect of molybdenum supply was consistent with existing knowledge regarding the role of molybdenum in the enzyme (17, 18). The failure of Evans and Nason (8) to observe an effect of molybdenum on the enzyme in young soya bean plants might have been due to the molybdenum reserves in large seeded legumes which may delay deficiency effects for a whole generation (12). It is also apparent that the effect of molybdenum may be small or negligible in regions possessing only minimal activity. It was found in cauliflower, as in fungi (19) that the enzyme as such is apparently not produced by tissues that have developed in the absence of sufficient molybdenum and cannot be immediately reactivated in disrupted cells. Work with fungi (19) has shown that 3 hours are required before the synthesis of new enzyme can be demonstrated after the addition of molybdenum to deficient mycelia. The activity in cauliflower was also increased when detached leaves were infiltrated with molybdate and increases have been observed since by Hewitt after 2 hours. In intact tomato plants the effect of adding molybdenum may be observed in two hours as nitrite production from the accumulated nitrate in the deficient tissues (22). A similar response to the addition of molybdenum to excised discs from molybdenum deficient cauliflower plants may also be inferred from experiments on respiration by Ducet and Hewitt (7). The effects of growing the plants with different sources of nitrogen also suggest adaptive features in the production of the enzyme. As with fungi (19), production of the enzyme was much greater in the presence of nitrate or nitrite than of ammonia, but was not completely suppressed by this last treatment as observed in the fungi (19).

Evans and Nason (8) suggested that the effect of light on nitrate reduction described by Burström (6) was consistent with the photochemical reduction of pyridine nucleotides by illuminated chloroplasts and their utilisation by nitrate reductase. The results obtained here show that exclusion of light from the plants may cause also a temporary loss of enzyme activity in extracts even when adequate reduced pyridine nucleotides are present. In this connection Stoy (23, 24) recently concluded that added riboflavin catalyses a photo-reduction of nitrate in irradiated wheat leaf extracts and provides a more effective electron donor for nitrate reductase in this system than reduced pyridine nucleotides. There are other examples also of the effects of light on enzyme production, as for example the observation of Appleman and Pyfrom (4) that red and blue light exert different effects on catalase production by etiolated barley plants.

Analogous changes to those observed in darkness and light were observed when air was excluded and re-admitted, but it cannot yet be concluded that they were in fact due to the same cause in both sets of treatments. Since this work was ended, Morton (15) observed independently that anaerobic conditions caused similar reversible changes in nitrate reduction capacity in fungi. He concluded that the enzymes responsible were not lost and resynthesised but were only reversibly masked by the anaerobic conditions. Removal of free carbohydrate from the medium or addition of ammonia produced similar reversible effects to those of anaerobic conditions. Lenhoff, Nicholas and Kaplan (14) have found that the relative proportions of flavin and haematin enzymes in *Pseudomonas fluorescens* also are determined by atmospheric conditions as well as by the available metals in the culture medium. Such changes contrast markedly with the tendency for enzymes such as invertase and peroxidase to persist even after extensive proteolysis in detached leaves kept in darkness as observed by Axelrod and Jagendorf (5). It may be concluded from this work that nitrate reductase is a highly labile enzyme in living cells as well as in vitro.

Work is now in progress, using this enzyme as a model, to investigate adaptive aspects of enzyme activity in higher plants as revealed by changes in nutritional or environmental conditions.

Summary

1. Cauliflower plants cv. Majestic were grown in sand culture with deficiency, normal or excess levels of molybdenum and with nitrogen supplied as nitrate, nitrite or ammonium sulphate.

2. Nitrate reductase was extracted by grinding leaves with sand in a mortar or by maceration in a high speed blendor with buffer solution at 0° C. These methods gave comparable activities but grinding in mechanically driven close fitting glass macerators produced low activities. The optimum pH was about 7.2 in phosphate buffer extracts.

3. The net nitrate reductase activity was maximal in mature leaves and was markedly lower in both senescent and rapidly expanding young leaves.

4. Extracts from leaves possessed greater net total activity than those from stems or petioles, and that of roots was extremely low. The specific activity per mg protein was much greater in petioles than in leaves or stems.

5. The nitrogen source also determined the level of nitrate reductase activity. This was high in plants given nitrate and usually high in those given nitrite. Plants given ammonium sulphate possessed markedly lower but not negligible activities.

6. In regions showing maximal activity, plants grown without molybdenum possessed much lower net total and specific nitrate reductase activities than the normal plants. In regions showing minimal activity these differences were small. Plants given excess molybdenum did not appear to possess more activity than normal plants. The effect of molybdenum supply mentioned above was greatest with plants given nitrate and least with those given ammonium sulphate.

7. Infiltration of molybdenum into leaves of deficient plants resulted in increases in nitrate reductase activity in 18 to 24 hours.

8. Nitrate reductase activity was greatly depressed by prolonged darkness during two to six days and recovered rapidly within a few hours of restoring the plants to light.

9. Exclusion of air for 48 hours also caused marked decreases in activity; and readmission of air led to rapid recovery in activity.

10. Changes in activity were not closely related to the total soluble protein content of the tissues. The distribution of the enzyme in plants grown with different molybdenum levels and nitrogen sources was markedly different from the distribution of extractable protein which was, however, similar to that of another enzyme—acid phosphatase.

The tenure of a Joint Overseas Studentship of the Consejo Superior de Investigaciones Scientificas Madrid and of the British Council, London, by M. I. Candela in 1953–55 and the grant of Sabbatic leave in 1955 to E. G. Fisher which respectively enabled them to participate in this work are gratefully acknowledged.

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TRANSLOCATION OF ORGANIC SUBSTANCES IN TREES. I. THE NATURE OF THE SUGARS IN THE SIEVE TUBE EXUDATE OF TREES[1]

MARTIN H. ZIMMERMANN

Cabot Foundation, Harvard University, Petersham, Massachusetts

Most investigators regard sucrose as the only form in which carbohydrates are translocated in plants. Some, however, suggest that hexoses are also translocated. It is beyond the purpose of this paper to list all these publications, a good bibliography will be found in the review of Crafts (1), and more recent publications are listed in the work of Ziegler (13).

One major difficulty in translocation work is the fact that possibilities of obtaining material that is actually transported are very limited. Chemical analyses based on tissue extractions yield substances which originate not only from sieve tubes but also from other types of cells in phloem and non-phloem tissues. There are only two ways of obtaining more or less pure sieve tube exudate. One method developed recently (7, 8) uses cut-off stylets of aphids which were feeding on the phloem. The other method, tapping the sieve tube system of trees by cutting into their bark, was first described by Th. Hartig (4) in 1860. It is this method that has been used in the present paper. There is plenty of evidence that the exudate obtained in this way is actually translocated material; Hartig found by microscopic observation that the exudate does come from the sieve tubes. This was later confirmed by Huber and Rouschal (5). Hartig observed that a second cut always yields sap if it is applied above the first one, below the first cut, however, a second cut usually failed to yield exudation (4). Sixty years later, Münch extended these studies and found that the sieve tube turgor is released up to several meters above a tapping cut (9). Ziegler supplied hexoses to the inner bark of trees (Robinia) and subsequently found them in the exudate below, but not above the point of application (13). The reason we referred to "more or less pure" sieve tube exudate is the possibility that exudate may elute traces of impurities from the cut surface during the tapping process.

Using chromatographic methods, Wanner found in 1953 that sucrose is the only sugar in the sieve tube exudate of *Robinia Pseudo-Acacia* L. and *Carpinus Betulus* L. (11). Testing exudate of 10 other European tree species, Ziegler also found only sucrose (13). However, in the course of a study on phloem translocation in white ash (*Fraxinus americana* L.), the results of which will be published elsewhere, it has been found that sucrose is by no means the only sugar in the exudate. It constitutes only 1/10 to 1/5 of the total sugar content. The other sugars identified are members of the raffinose family: raffinose, stachyose verbascose. In an attempt to learn whether white ash is an exceptional case, the sieve tube exudate of 16 tree species was collected and analyzed. The results of this survey are presented here.

Experimental

Sieve tube exudate was obtained by cutting into the inner bark of the tree with a sharp knife. In older trees it was necessary to remove the dead bark with an axe first. The tapping cut must penetrate the region of the functioning sieve tubes, which are located a fraction of a millimeter outside the cambium. Too deep a cut, however, severs the xylem, which, when it is under negative pressure, will immediately draw the exudate back with a hissing sound. The samples were either directly transferred onto the paper chromatograms or collected in a vial and immediately frozen in a Thermos bottle containing an ice-salt mixture. Exudate from white ash and red oak was collected from many individual trees during the summer and fall of 1955 and 1956. The samples from all other tree species were collected during the month of September 1956.

[1] Received December 18, 1956.

Paper chromatography was carried out using Whatman No. 1 paper in a descending procedure. The partitioning solvent was n-butanol : acetic acid : water in various proportions as described below. A color reaction was used to differentiate fructose-free and fructose-containing oligosaccharides on the same filter paper. The chromatograms were first dipped into the aniline hydrogen oxalate reagent of Partridge (10) which was prepared by dissolving 0.93 gm aniline in 50 ml 96 % ethanol and mixing the solution with an equal volume of 0.2 M oxalic acid in 96 % ethanol. The only modification of Partridge's method was the use of an alcoholic instead of an aqueous acid solution, and the chromatograms were dipped instead of being sprayed. The chromatograms were then heated for a few minutes at 110° C. Aldoses appeared first as a brown coloration, ketoses later. As soon as fructose started to react the paper was removed from the oven and subsequently dipped into a resorcinol reagent which contained equal volumes of 0.5 % resorcinol in acetone and 5 % HCl in 96 % ethanol. The paper was then heated at 70° C for 10 to 15 minutes until the red color due to fructose appeared. If the temperature in the oven is evenly distributed the reaction can equally well be carried out at higher temperature in a shorter time. This procedure causes fructose-free sugars to appear as brown spots and fructose-containing sugars as bright red spots on the same paper.

The oligosaccharides were identified by running side by side a sample of the known sugar, a sample of the exudate and a mixture of both for several days with a partitioning solvent of high resolution (e.g., n-butanol : acetic acid : water = 4 : 1 : 1 or 6 : 1 : 1 v/v/v). In other experiments, the exudate was placed onto the origin of the chromatogram and sprayed with a solution of 1 gm invertase concentration in 10 ml of water, after masking the rest of the chromatogram in such a manner that the sprayed invertase solution reached the exudate only. The resulting sugars were compared with pure reference sugars. The separation of the sugars shown in figure 1 was achieved with a butanol solvent of high water content (n-butanol : acetic acid : water = 3 : 3 : 2 v/v/v). This was used for the rapid separation of higher oligosaccharides. With a solvent of high resolution (low water content) sucrose runs off the paper before the oligosaccharides of high molecular weight are properly separated. Many repetitions under a variety of conditions gave the same results.

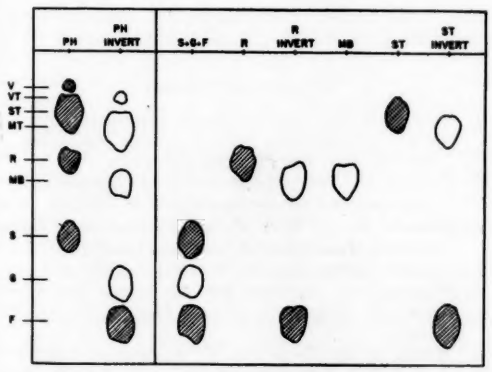

FIG. 1. One dimensional, descending paper chromatogram of untreated sieve tube exudate of white ash (PH), sieve tube exudate treated with a fructosidase (PH INVERT), and reference sugars (right of line). Partitioning solvent, n-butanol : acetic acid : water = 3 : 3 : 2 v/v/v, 24 hours. With the treatment described, fructose-containing sugars show up bright red (hatched), fructose-free sugars brown (open). Sugars: V = verbascose, VT = verbascotetraose, ST = stachyose, MT = manninotriose, R = raffinose, MB = melibiose, S = sucrose, G = glucose, F = fructose.

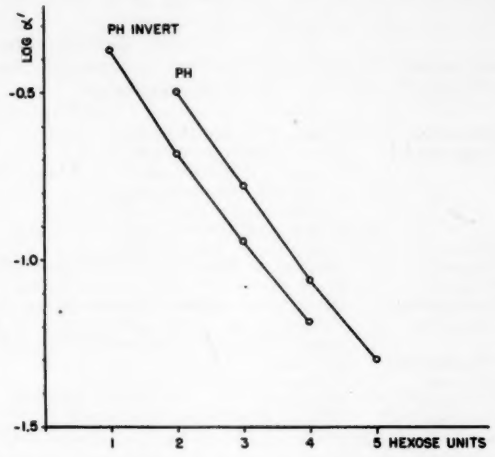

FIG. 2. Mobility of the sieve tube oligosaccharides on the filter paper. PH = sieve tube exudate of white ash, PH INVERT = same, treated with a fructosidase. $\alpha' = R_f/1 - R_f$ = partition function calculated according to French and Wild (3). Partitioning solvent, n-butanol : acetic acid : water = 3 : 3 : 2 v/v/v, 15 hours descending.

Although no reference sample of verbascose was available, the relative mobility of the sugars on filter paper strongly suggests that the slowest moving oligosaccharide in the sieve tube exudate of white ash is verbascose. It has been found by French and Wild (3) that the papergram mobilities of a true series of oligosaccharides, when expressed as logarithm of the partition function $\alpha' = R_f/1 - R_f$, and plotted against the molecular size, fall on a curve which approaches a straight line at low R_f-values. We preferred the descending technique but used a solvent of high water content which yields good separation of the higher oligosaccharides before the solvent reaches the end of the paper. Thus the R_f-values can be measured directly, and it is not necessary to calculate single as-

TABLE I

TRANSLOCATION SUGARS IN TREES

FAMILY	NAME OF TREE *	SUCROSE	RAFFINOSE	STACHYOSE	VERBASCOSE
Salicaceae	Aspen (*Populus tremuloides* Michx.)	+++	tr	tr	–
Fagaceae	Beech (*Fagus grandifolia* Ehrh.)	+++	tr	–	–
	Chestnut (*Castanea dentata* (Marsh.) Borkh.)	+++	tr	–	–
	White oak (*Quercus alba* L.)	+++	–	–	–
	Chestnut oak (*Quercus Prinus* L.)	+++	–	–	–
	Red oak (*Quercus rubra* L.)	+++	–	–	–
Ulmaceae	Elm (*Ulmus americana* L.)	++	++	++	–
Magnoliaceae	Tulip tree (*Liriodendron Tulipifera* L.)	+++	tr	tr	–
Rosaceae	Black cherry (*Prunus serotina* Ehrh.)	+++	tr	–	–
Leguminosae	Black locust (*Robinia Pseudo-Acacia* L.)	+++	–	–	–
Aceraceae	Striped maple (*Acer pensylvanicum* L.)	+++	tr	–	–
	Sugar maple (*Acer saccharum* Marsh.)	+++	–	–	–
Rhamnaceae	Buckthorn (*Rhamnus cathartica* L.)	+++	tr	tr	–
Tiliaceae	Linden (*Tilia americana* L.)	+++	+	+	–
Nyssaceae	Black gum (*Nyssa sylvatica* Marsh.)	+++	tr	–	–
Oleaceae	White ash (*Fraxinus americana* L.)	++	++	+++	tr

The total sugar concentration of sieve tube exudate is usually between 10 and 25 %, varying from tree to tree and fluctuating somewhat diurnally and seasonally. Thus the sign +++ corresponds to more than 10 %, ++ to about 2 to 10 %, + to 0.5 to 2 %, tr (trace) to less than 0.5 %, and – (not found) to less than about 0.1 %.

* The nomenclature follows Gray's Manual of Botany, 8th ed., 1950.

cent R_f-values from multiple ascent values. Figure 2 shows that the series sucrose–raffinose–stachyose and their fructose-free equivalents do form the curves that have been found by French and Wild (3), and the positions of the fourth spot and its fructose-free form indicate that the oligosaccharides in question are verbascose and verbascotetraose respectively.

RESULTS

The sieve tube exudate of 16 tree species of 11 families has been analyzed. Non-reducing oligosaccharides are the only sugars that have been found. Sucrose appears to be the main translocation form, but the sieve tube exudate of many species also contains higher oligosaccharides. These have been identified as members of the raffinose family, as described in the experimental part and in figures 1 and 2. The chemistry of this group of sugars has been comprehensively reviewed by French (2).

Table I shows the results in detail. The translocation sugars found in white ash are mainly galactose containing oligosaccharides. These sugars also are important constituents in elm and linden. The fact that traces of raffinose occur in the exudate of so many species investigated suggests that the sugars of the raffinose family (sucrose, raffinose, stachyose, verbascose) are the transport sugars. Their occurrence in a wide variety of plants (12) shows their relative importance. They have also been found in many herbaceous plants (Mentha, Stachys, etc.) where they probably occur in the sieve tube system, as they do in trees. This remains to be proved, however.

SUMMARY

The sugar content of the sieve tube exudate of 16 tree species of 11 families has been analyzed. Only non-reducing oligosaccharides of the raffinose family have been found. Sucrose is the major transport sugar, but in some tree species most (white ash) or part (elm, linden) of the sieve tube sugars are in the form of higher oligosaccharides, raffinose and stachyose.

The writer wishes to express his appreciation to Prof. Hugh M. Raup, director of the Harvard Forest, for making available the laboratory facilities for this work, and to Mr. Benjamin B. Stout for making it possible to obtain samples from trees at the Harvard Black Rock Forest. Prof. Kenneth V. Thimann kindly consented to read and criticize the manuscript.

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RESPIRATION OF THE MYCELIA AND MITOCHONDRIA OF THE FILAMENTOUS WATERMOLD, ALLOMYCES MACROGYNUS[1,2]

BRUCE A. BONNER[3] AND LEONARD MACHLIS
Department of Botany, University of California, Berkeley, California

The immediate purpose of this investigation of respiration in the watermold *Allomyces macrogynus* was to provide a basis for the ultimate explanation of the effect of various nutritional conditions on the utilization of mannose and fructose by this mold (28, 29, 37). Its primary significance, however, is the isolation from one filamentous fungus of mitochondria which oxidize the various intermediates of the Krebs cycle. Only mitochondria from yeast, among the fungi, have been shown to contain an organized system associated with mitochondria for carrying out these reactions (24, 32). The reader is referred to Bonner (4) for a comprehensive review of pathways of carbohydrate metabolism in the fungi and the related actinomycetes.

Materials and Methods

Growth and Preparation of the Fungus: The organism used was the Burma 1 Da strain of *Allomyces macrogynus* (10). It was grown in liquid, agitated culture in 50 ml of medium in 125-ml Erlenmeyer flasks as described by Machlis (27). The complete medium, as used in this study, is the same as the minimal medium of Machlis (27) except for the addition of 10^{-4} M L-glutamic acid. Low-sugar medium differs only in that the concentration of glucose is 0.05 % instead of 0.5 %. Each flask of medium was inoculated with 0.5 or 1.0 ml of mitospores and then incubated in the dark (except when room lights were turned on for observations) on a rotary shaker at 25° C for 48 to 96 hours. The mitospore inoculum was prepared as previously described (26).

For the manometric determinations, the flasks of spherical plants were emptied over a wire screen or nylon cloth, the occasional large plants removed, and the remaining retained plants rinsed with three or four washes of 0.04 M potassium phosphate buffer at pH 7.0. The rinsed plants from the requisite number of flasks were then composited, suspended in the solution to be used in the vessels and transferred to the Warburg vessels with a calibrated pipette having an opening approximately 5 mm in diameter.

Preparation of Mitochondria: Initial trials to obtain mitochondria were unsuccessful using homogenizers of the Ten-Broeck and Potter-Elvehjem type, carborundum of several mesh sizes, and washed sand. At the suggestion of Dr. V. W. Cochrane, ground Pyrex glass was tried and mesh sizes 20 through 50 were found to work well. The screened glass was washed with acid followed by distilled water until all acid was removed. It was then oven-dried.

For a typical experiment 40 flasks of plants grown in complete medium for 80 to 96 hours were used. They were removed from the growth medium and washed on a nylon cloth over a Büchner funnel with approximately 500 ml of cold 0.04 M potassium phosphate buffer (pH 7.0 or 7.4). Excess buffer was pressed from the plants which were then ground in a cold room with 50 to 60 gm of ground glass and a final total of 150 to 180 ml of a solution containing 0.5 M sucrose, 0.1 M potassium phosphate, and 0.001 M potassium ethylenediamine tetraacetate at a pH o 7.0 or 7.4. Both pH 7.0 and 7.4 gave identical results.

The plants, glass, and about a third of the buffer solution were ground in a cold mortar on ice for 6 to 8 minutes until a smooth paste was formed. More buffer was added, mixed with the paste and the liquid strained off through a nylon net on a funnel into test tubes immersed in ice. The glass and debris were returned to the mortar with the remainder of the buffer, reground for 2 or 3 minutes, then filtered through the net again, and the easily removable liquid squeezed out. The crude, filtered homogenate was then centrifuged for 10 minutes at $500 \times g$ in a refrigerated angle centrifuge held at $-2°$ to $+2°$ C. This removed the cellular debris and all but the finest of the glass particles. The supernatant was decanted into more

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[3] During the progress of this work the first author held University Fellowships and a terminal National Science Foundation Fellowship. The research was submitted by him in partial fulfillment of the requirements for the Ph.D. in Botany at the University of California at Berkeley. Present address: Laboratoire de Génétique, Physiologique du CNRS, Gif-sur-Yvette, S. e. O., France.

50-ml tubes and centrifuged for 15 minutes at 10,000 × g. The next supernatant was discarded and approximately 10 ml of sucrose-buffer solution added to each tube. This solution differed from that used in grinding only in that the phosphate concentration was lowered to 0.01 M. The particles were loosened with a plastic pestle and resuspended in a small glass tissue homogenizer with the plastic pestle rotated by hand. This suspension was consolidated into two tubes and again centrifuged at 10,000 × g for 15 minutes. The supernatant was decanted and the particles resuspended as above in 5 to 10 ml of a solution of 0.5 M sucrose, 0.001 M potassium EDTA, and 0.001 M potassium phosphate buffer (pH 7.0 or 7.4). One-half-ml aliquots of the cold suspension were then added to the Warburg vessels containing the buffer, cofactors, and substrates in the main compartment. The vessels were put on the Warburg manometers as quickly as possible and placed in the water bath. After an 8- to 10-minute equilibration period the taps were closed and readings taken for a period usually of thirty minutes. An aliquot of the particulate suspension was taken for nitrogen determination by the micro-Kjeldahl method.

MANOMETRIC AND CHEMICAL DETERMINATIONS: Manometric determinations were by the direct method of Warburg with corrections for the retention of CO_2 in the buffer (38). The temperature was 25 or 30° C in experiments with intact plants and 30° C with mitochondria.

Manometric data involving absolute rates of gas exchange of intact plants are expressed on the basis of the dry weight of plant material in each Warburg vessel at the end of the experiment. This was done because no satisfactory method was found for introducing uniform samples into the vessels. Fragmenting the mycelium destroys most of the respiratory activity (17), presumably because *A. macrogynus* is a coenocyte and therefore will not break up into the short chains of cells Darby and Goddard (8) were able to use with *Myrothecium verrucaria*. This method of expressing results has the disadvantage that within an experiment different treatments may differentially affect the final dry weight.

In certain experiments uniformly labeled glucose was fed to intact plants for varying periods of time. After the exposure, the plants were rinsed quickly with distilled water and dropped into 80 % ethanol (4 to 6 mg dry weight of plants to 20 ml ethanol). The plants were centrifuged down, re-extracted with approximately 20 ml of 20 % ethanol, recentrifuged and the two extracts combined. The combined extract was concentrated first in a vacuum still and then by blowing nitrogen gas over it. The extracts were examined by the chromatographic and radioautographic methods (1) used by the Bio-Organic Group of the Radiation Laboratory of the University of California at Berkeley where this phase of the work was done through the courtesy of Dr. M. Calvin. The uniformly labeled glucose-C^{14} was prepared photosynthetically. It and the Polidase-S (Schwarz Laboratories, Inc.), an acid phosphatase preparation, were kindly supplied by Dr. J. A. Bassham of the Bio-Organic Group named above.

EXPERIMENTAL RESULTS

GENERAL CHARACTERISTICS OF RESPIRATION: Preliminary experiments on the oxygen uptake of intact mycelia indicated that the Q_{O_2} was depressed when the dry weight of mycelium per Warburg vessel exceeded the low amount of approximately 8 mg. It appeared that excess plant material prevented the free movement of liquid during the shaking and hence the access of oxygen to the plants. To clarify the relation, mycelium of varying amounts of dry weight was added to vessels with liquid volumes of 1.5, 2.7, and 4 ml yielding a range of ratios of dry weight in mg to liquid volume in ml as indicated in figure 1. As the results show, the measured Q_{O_2} decreases at ratios greater than 2.5. When the mean Q_{O_2} for those flasks with ratios greater than 2.5 was compared with the mean of those with ratios of 2.0 or less, the means were significantly different by the t test at a level of 99 %. Similar results were obtained at a temperature of 30° C. When the ratio was well above 2.0, increasing the shaking rate increased the Q_{O_2}; with ratios less than 2.0, there was no effect of an increased rate of shaking. In all subsequent experiments a ratio of less than 2.0 was used.

The composition and pH of the suspension medium are known to profoundly affect both the endogenous respiration and the respiratory responses to added substrates and inhibitors. To test the effect of the composition of the suspension medium, the growth medium excluding organic components was used as the control.

If only the phosphate buffer was used, there was a rapid decline of the endogenous Q_{O_2} (table I) Upon adding back the omitted inorganic constituents, the maintenance of the Q_{O_2} equal to the control was found to depend on the presence of the calcium ion. Magnesium did not substitute for the calcium and calcium could not reverse the decline in Q_{O_2} if added after 40 minutes in the phosphate buffer alone.

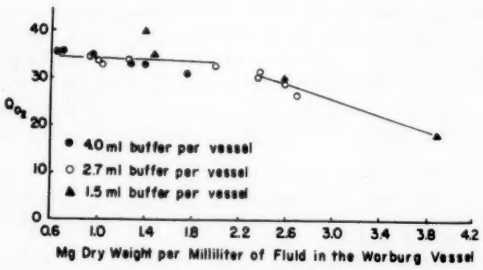

FIG. 1. The relationship of dry wt and fluid vol to the Q_{O_2}. The plants were grown for 78 hrs in complete medium and suspended in 0.5 % glucose, 0.015 M potassium phosphate at pH 7.0, 0.5 mM $CaCl_2$ and 0.5 mM $MgCl_2$.

TABLE I

THE EFFECT OF THE COMPOSITION OF THE SUSPENDING MEDIUM ON THE RESPIRATION OF ALLOMYCES MACROGYNUS GROWN 83 HRS IN COMPLETE MEDIUM

SUSPENDING MEDIUM	Q_{O_2}
Control, inorganic constituents of the growth medium	23
0.015 M Potassium phosphate, pH 7.0	9
0.001 M $CaCl_2$ and 0.015 M potassium phosphate, pH 7.0	25
0.001 M $MgCl_2$ and 0.015 M potassium phosphate, pH 7.0	12
0.0005 M $CaCl_2$, 0.0005 M $MgCl_2$ and 0.015 M potassium phosphate, pH 7.0	24
Distilled water	19

The effect of the pH of the suspending medium was assessed using phosphate and tartrate buffers. Although the two buffers were tested in separate experiments, the plants were comparable; hence, the results are presented in a single graph (fig 2). A pH of 4.6 or less markedly inhibits respiration; however, about 20 % of the growth of the plants is made at a pH below 4.6 because the pH of the medium drops continuously during growth. In the experiment just described the pH of the growth medium at the time the plants were used was 6.0. If, however, the plants were permitted to grow until the culture medium reached pH 4.6, then transfer to a suspension medium at 4.6 was not inhibitory. Thus, if the plants accommodate to the low pH by growth, the pH is not inhibitory.

Allomyces, like many other fungi, has a high endogenous respiration which in the past has interfered with respiratory studies (17, 41). Figure 3 shows, for plants 61, 71 and 86 hours old, the progress of the endogenous respiration and the effect of adding potassium acetate. The endogenous activity declines, more so in the younger plants, and acetate does little more than prevent the decline.

In an attempt to reduce the endogenous respiration and elicit larger responses to added substrate, plants were starved by holding them in buffer solution on the shaker. There was no increase in response upon adding substrate. The enzymatic capacities apparently declined as rapidly as the endogenous Q_{O_2}. A somewhat different approach was more successful. Plants were grown in low-sugar medium to prevent the accumulation of reserve foods. Figure 4 shows that the endogenous respiration was considerably reduced in the older plants and that the addi-

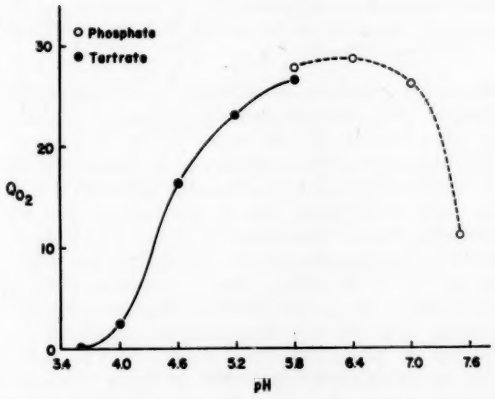

FIG. 2. The effect of pH on respiration. The plants were grown for 84 to 86 hrs in complete medium and suspended in 0.015 M potassium phosphate or potassium tartrate adjusted to the indicated pH values with KOH or HCl, 0.5 mM $MgCl_2$ and 0.5 mM $CaCl_2$.

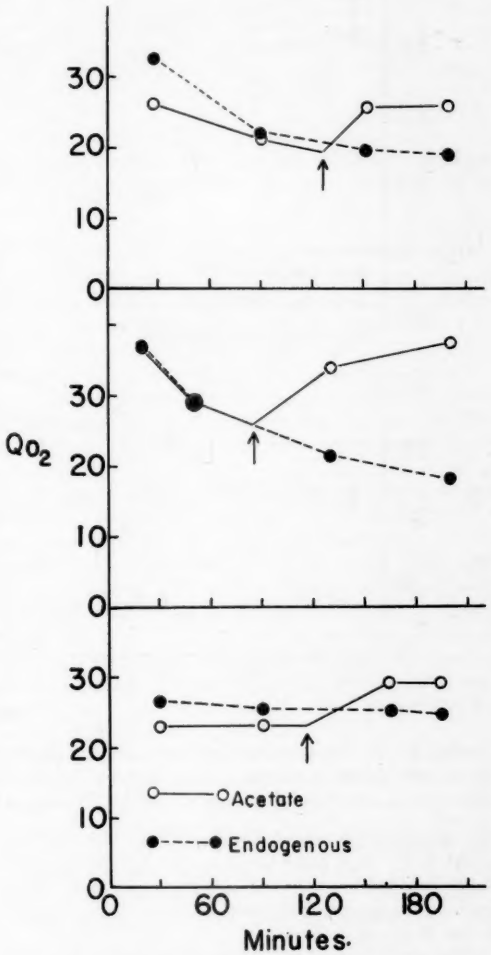

FIG. 3. The endogenous respiration and the effect of added substrates. Plants grown for 61 (*upper*), 71 (*middle*) and 86 (*lower*) hrs in complete medium and suspended in the inorganic components of the complete medium. Acetate conc, after tipping, was 0.01 M.

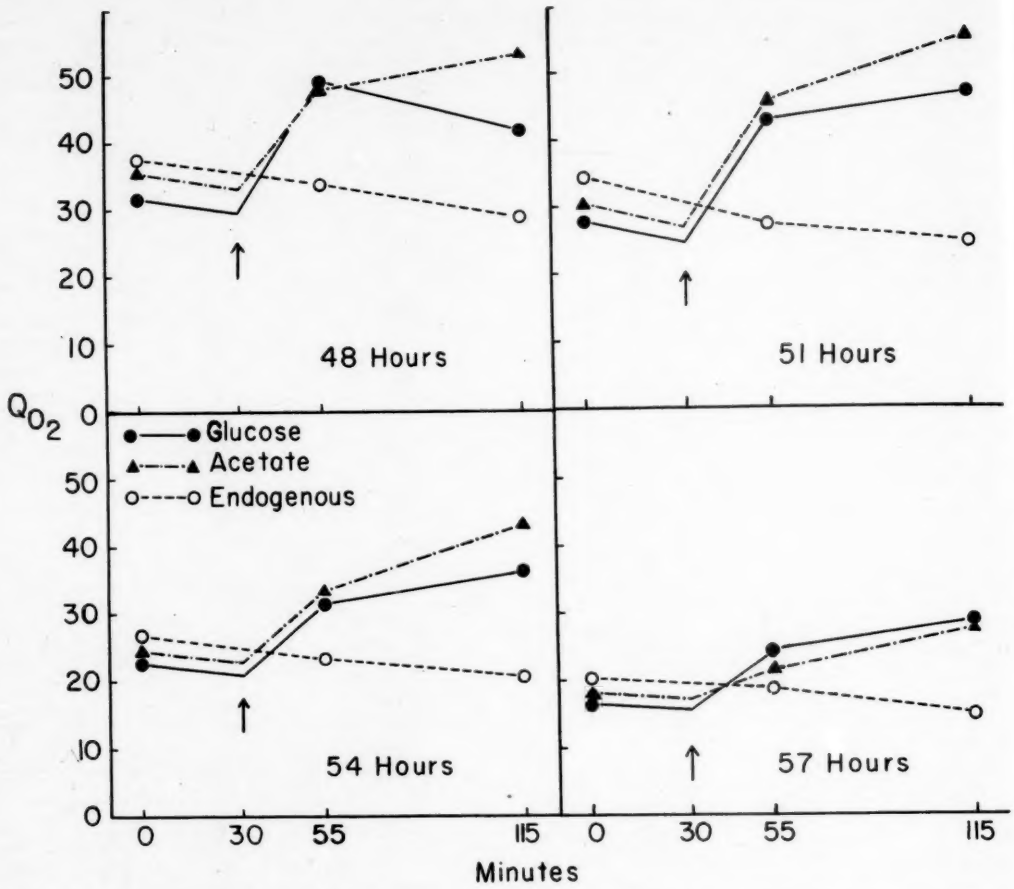

FIG. 4. The endogenous respiration and the effect of added substrates on mycelium grown in low-sugar medium. Plants were grown in low-sugar medium for the times indicated and suspended in the growth medium less glucose. After tipping, the conc of glucose was 2.75 mM and that of acetate was 8.25 mM.

tion of glucose increased the Q_{O_2} 138 to 158 % of the initial rate. Still older plants could not be used because they become covered with mitosporangia which discharge large numbers of mitospores upon transfer to the Warburg vessels.

The R.Q. of the endogenous respiration was consistently 0.8 to 0.9. The addition of glucose raised the R.Q. to slightly over 1.0. Actual values with mycelium grown in complete medium for 61 to 63 hours and then suspended in fresh complete medium were 1.22 with sugar and 0.88 without during the first 70 minutes and 1.09 and 0.82 during the first 130 minutes. In view of copious oil globules in the mycelium, there may possibly be fat oxidation in the absence of glucose and fat synthesis in the presence of glucose.

THE EFFECTS OF INHIBITORS: The effects of the five inhibitors, iodoacetate, fluoride, arsenite, fluoroacetate, and malonate, were studied. The most likely enzymes inhibited by these poisons are triosephosphate dehydrogenase by iodoacetate (19), enolase by fluoride (39), pyruvate and α-ketoglutarate oxidation by arsenite (20, 21, 33), aconitase by fluoroacetate (31) and succinic dehydrogenase by malonate (34, 35).

The results with iodoacetate are compiled in table II. The endogenous rate of low-sugar plants was measured over a 30-minute period and served as the basis for comparison between the various treatments. At the end of this period either substrate in buffer or buffer alone was added from a sidearm. After 15 minutes a second rate measurement was made over the 50- to 80-minute time period. At the end of this period inhibitor in buffer or buffer alone was tipped in from the other sidearm and another rate measurement made over the period 110 to 140 minutes. Changes are calculated as a percentage of the initial endogenous Q_{O_2} to eliminate differences between Q_{O_2} values based on the final dry weights of the plants which probably reflect increases in dry weight

TABLE II

IODOACETATE INHIBITION OF RESPIRATION IN THE PRESENCE AND ABSENCE OF ADDED SUBSTRATES *

MIN	ENDOGENOUS				GLUCOSE, 2.75 MM				ACETATE, 8.25 MM			
	CONTROL		IODOACETATE, 0.001 M		CONTROL		IODOACETATE, 0.001 M		CONTROL		IODOACETATE, 0.001 M	
	Q_{O_2}	%	Q_{O_2}	%	Q_{O_2}	%	Q_{O_2}	%	Q_{O_2}	%	Q_{O_2}	%
0–30	31.7	100	32.3	100	27.3	100	34.9	100	25.9	100	29.8	100
50–80	29.5	93	30.7	95	39.4	144	50.8	145	37.2	144	43.1	145
110–140	24.3	77	5.2	16	39.2	144	5.4	15	44.0	170	35.9	120
Inhibition, %		79				90				29		

* The main compartment contained 3.2 ml of growth medium less sugar with 3.6 to 6.8 mg dry wt of plants grown 52 hrs. Potassium acetate and iodoacetate were in 0.4 ml of buffer in separate sidearms. All values based on the average of 2 or 3 vessels.

during the experiment when substrate was present and decreases when substrate was absent. The percent inhibition is calculated using the comparable values from the control vessels lacking iodoacetate.

Iodoacetate at 10^{-3} M inhibits both the endogenous respiration and the increased respiration made possible by glucose. Acetate effectively bypasses the iodoacetate inhibition. When lower levels of iodoacetate are used (10^{-4} and 5×10^{-4} M, causing 59 and 89 % inhibition, respectively) the bypassing of the inhibition by acetate is virtually complete.

These levels of iodoacetate are in the concentration range which causes inhibition of triosephosphate dehydrogenase (19). Much higher levels are required to inhibit succinic dehydrogenase (36) while alcohol dehydrogenase inhibition (9) would appear not to be involved since the organism neither uses nor produces ethanol (18). The respiration of glucose thus appears to be blocked at triosephosphate dehydrogenase by iodoacetate. The insensitivity of the acetate metabolism to iodoacetate inhibition supports this interpretation.

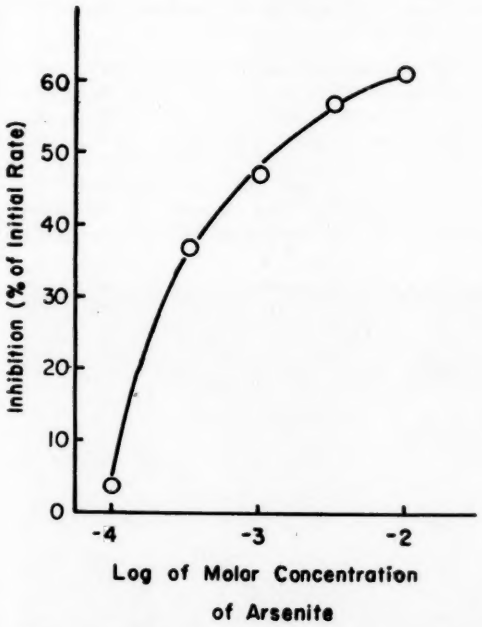

FIG. 5. Arsenite inhibition of oxygen consumption in the presence of glucose. Plants grown in low-sugar medium for 58 hrs and suspended in complete medium containing 0.1 % glucose.

Sodium fluoride was added to low-sugar plants suspended in complete medium with and without the glucose. The Q_{O_2} after 35 minutes exposure to the fluoride was inhibited 27 and 18 % by 0.01 and 0.005 M sodium fluoride, respectively, in the absence of glucose and 76 and 25 %, respectively, in the presence of glucose. Thus, the endogenous respiration is relatively insensitive to fluoride, while glucose metabolism is strongly inhibited. These data are consistent with the interpretation that enolase is involved in glucose metabolism.

The effect of the arsenite on low-sugar plants suspended in medium containing 0.1 % glucose is shown in figure 5. Fifty percent inhibition (calculated as the percent reduction of the initial rate) is effected by approximately 10^{-3} M arsenite, indicating the respiration of glucose involves the pyruvate and α-ketoglutarate oxidase systems.

Fluoroacetate inhibits both the endogenous and the glucose-stimulated respiration as would be expected if respiration is mediated by a typical Krebs

TABLE III

MALONATE INHIBITION *

TREATMENT	Q_{O_2} AS % OF CONTROL	% INHIBITION
Control	100	0
0.005 M Malonate	59	41
0.01 M Malonate	31	69
0.05 M Malonate	33	67

* The plants were grown in complete medium for 84 hrs and suspended in 0.01 M potassium tartrate, 0.5 mM $MgCl_2$, 0.5 mM $CaCl_2$ at pH 4.8 in the Warburg vessels.

cycle. The endogenous rate is reduced 66 % (from a Q_{O_2} of 26.5 to 9.4) by 0.001 M sodium fluoroacetate and 74 % by 0.005 M while the rate with 0.5 % glucose present is reduced 40 % (Q_{O_2} of 41.1 to 24.7) by 0.001 M and 47 % by 0.005 M.

Malonate inhibition and its reversal by succinate, while among the most specific indicators of Krebs cycle activity, is very sensitive to pH. In table III are the results of an experiment in which plants grown in complete medium were suspended in potassium tartrate-$MgCl_2$-$CaCl_2$ buffer at pH 4.8 with several concentrations of the sodium salt of malonate adjusted to pH 4.8 with HCl. The pH at the end of the experiment was 4.9 to 4.95. The inhibition observed could not be reversed by succinate since the latter was inhibitory at concentrations greater than 0.01 M. As will be shown later, a succinate to malonate ratio as high as 10 permits only 32 % of the control respiration of isolated mitochondria. The use of this or higher ratios with intact mycelia results in nonspecific inhibition by the succinate.

STUDIES WITH LABELED GLUCOSE: In the first experiment, plants grown 52 hours in low-sugar medium were washed and added to the Warburg vessels so that each vessel contained 4 to 6 mg dry weight of plant material in 3.6 ml of growth medium lacking glucose. There was no KOH in the center well. After an equilibration period of 10 minutes, the sidearm contents consisting of 0.45 ml of buffer containing 4.5 μc of glucose-1-6-C^{14} (63 μgm) was tipped in. After 5 and 20 minutes the plants were collected, rinsed quickly with distilled water and dropped into 20 ml of 80 % ethanol.

The distribution of the radioactivity after the 5- and 20-minute periods of metabolism is shown in figure 6 and quantitatively in table IV. In a period of 5 minutes the radioactive carbon from glucose was distributed among a number of sugar phosphates, nucleotides, and amino acids. After 20 minutes the pattern was much the same with some redistribution of the label away from the sugar phosphates.

TABLE IV

DISTRIBUTION OF RADIOACTIVITY ON CHROMATOGRAMS OF BREAKDOWN PRODUCTS OF GLUCOSE

Spot	Compound	5-Min exposure to glucose		20-Min exposure to glucose	
		Cpm *	% of total recovered	Cpm *	% of total recovered
A	Uridine diphosphate glucose, etc.	2400	6.6	1370	4.7
B	Nucleotides	2770	7.7	6090	20.7
C	Nucleotides	1800	5.0	1600	5.4
D	Hexosemonophosphates	6700	18.5	1600	5.4
E	Phosphoglyceric acid	3700	10.2	1700	5.8
F	Triosephosphate	5300	14.6	2400	8.2
G	Pentose phosphates	550	1.5	....	...
H	Phosphoenolpyruvate	1700	4.7	880	3.0
I	Aspartic acid	470	1.3	2600	8.8
J	Isocitrate	....	...	270	0.9
K	Malate	340	0.9	500	1.7
L	Glutamic acid	660	1.8	2700	9.2
M	Alanine	8100	22.4	7200	24.5
N	...	300	0.9	....	...
Others	...	1400	3.8	500	1.7

* Counts per minute are corrected for background but are not corrected for coincidence which causes low values over 5000 cpm. Because of high activities, counting was done with an aluminum foil filter between the spot and the detector tube resulting in a 9-fold reduction of the counts actually obtainable with the geometry of the tube used.

The compounds listed in table IV were identified by eluting the spots, treating with polidase, and chromatographing the hydrolysates with carriers (A, B, C, and G) or by position and spray reactions (spots E, F, H, I, J, K, L, and M). Spots A, B, and C absorbed ultraviolet light and were tentatively identified as nucleotides. Spot A gave rise to mannose, glucose, ribose, and uridine as well as some unidenti-

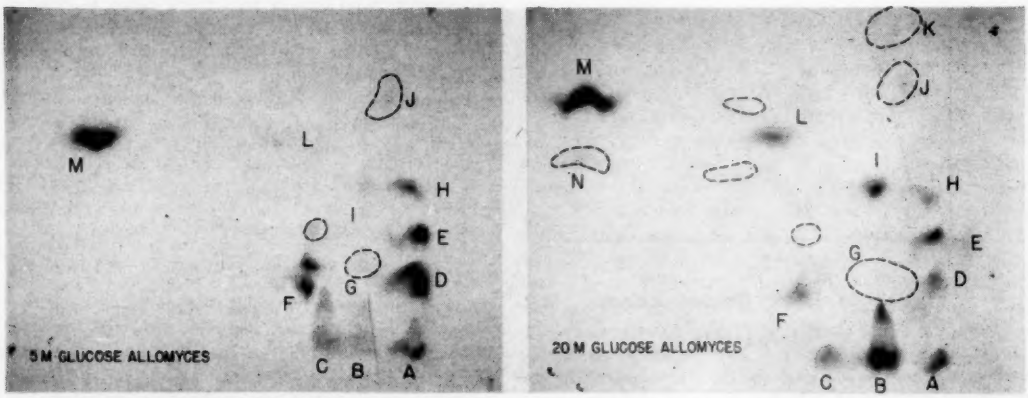

FIG. 6. Radioautograms of alcohol extracts of Allomyces exposed to uniformly labeled glucose-C^{14} for 5 min (*left*) and 20 min (*right*).

fied substances; from spot B was identified uridine, ribose, and possibly xanthosine or guanosine; and from spot C uridine, ribose and probably xanthosine or guanosine. Spot D, the hexosephosphate area yielded mannose, fructose, glucose and possibly sedoheptulose or galactose. The pentose phosphate area, G, yielded ribose and arabinose and several unidentified spots.

In table IV it may be seen that between 5 and 20 minutes there is a marked decline in the radioactivity in sugar phosphates (D), triosephosphate (F), and phosphoglyceric acid (E), while the radioactivity in glutamic acid (L) and aspartic acid (I) increases. This is suggestive of a flow of radioactive carbon from glycolytic intermediates into by-products of the Krebs cycle with the longer incubation period. It is also of interest to note the relatively large amounts of activity recovered in the nucleotides (spots A, B, and C).

A second experiment with radioactive glucose was designed to test the effects of sodium fluoroacetate and sodium iodoacetate upon the distribution of the radioactivity. Plants were exposed to 5 mM sodium fluoroacetate, 0.5 mM sodium iodoacetate, or to buffer for 10 minutes, after which labeled glucose was tipped in. Forty minutes later the plants were washed and extracted. The activity recovered was too small to make dark enough radioautograms after 4 weeks exposure for effective photographic reproduction. With the control, 45 % of the recovered activity was in glutamic acid, aspartic acid, and alanine. In contrast, the chromatograms of plants treated with iodoacetate showed no activity in these spots although there was sufficient inactive material to give positive ninhydrin reactions. No compounds beyond the triosephosphate stage were recovered. The chromatograms from the plants treated with fluoroacetate differed from the above chromatogram only in having a large, relatively heavily labeled spot corresponding to J in figure 6, presumably citrate. Citrate accumulation is characteristic of fluoroacetate poisoning (6). These results served to confirm the usual interpretations of the inhibitions observed manometrically.

TABLE V

OXIDATION OF ORGANIC ACIDS BY MITOCHONDRIA OF ALLOMYCES *

SUBSTRATE	ADDITIONAL COFACTORS	$Q_{O_2}(N)$
Succinate	.....	360
Fumarate	.....	98
Citrate	0.1 mM TPN	170
Cis-Aconitate	0.1 mM TPN	286
Isocitrate	0.1 mM TPN	306
α-Ketoglutarate	.....	137
α-Ketoglutarate	0.1 mM ThPP 0.25 mg CoA	291
Acetate	0.1 mM ThPP 0.25 mg CoA	−37 **
Malate	0.1 mM ThPP 0.25 mg CoA	−29 **
Malate (2 mM)	0.1 mM ThPP 0.25 mg CoA	0
Pyruvate	0.1 mM ThPP 0.25 mg CoA	20
Pyruvate +2 mM Malate	0.1 mM ThPP 0.25 mg CoA	247
None	.....	−43 **

* The Warburg vessel contained 3.0 ml of reaction mixture, mitochondria equivalent to 0.28 mg nitrogen, and other components in the following concentrations: 0.35 M sucrose, 1 mM $MgCl_2$, 0.01 mM cytochrome c, 0.1 mM DPN, 1 mM ATP, 16 mM PO_4 pH 7.4, 10 mM glucose, and 20 mM substrate (unless otherwise noted).

** Slight positive pressures developed in the manometers.

TABLE VI

EFFECT OF COFACTORS ON THE SUCCINOXIDASE SYSTEM *

SYSTEM	$Q_{O_2}(N)$
Complete	378
− Cytochrome c	138
− DPN	404
− ATP	292
− $MgCl_2$	361
− Succinate (endogenous)	−9
+5 mg Hexokinase	378

* The complete system contained these substances in the concentrations indicated: 0.02 M succinate, 1 mM $MgCl_2$, 0.01 mM cytochrome c, 0.1 mM DPN, 1 mM ATP, 0.35 M sucrose, 10 mM glucose, 17 mM potassium phosphate buffer pH 7.4, and mitochondria equivalent to 0.23 mg nitrogen. The center well contained 0.2 ml 20 % KOH.

RESPIRATION OF MITOCHONDRIA: Washed suspensions of particles prepared, as described under Methods and Materials, from homogenates of Allomyces grown in complete medium will oxidize most of those intermediates tested as shown in table V. The conditions used in this experiment were set up without a knowledge of optimum conditions for the various reactions and, thus, may not have resulted in maximum rates. Significantly higher rates have been measured in other experiments with most of the acids.

The rates of oxidation of fumarate and pyruvate are low, while malate is not appreciably oxidized. However, when malate and pyruvate are both present, the oxygen consumption is enhanced several fold over that observed with either substrate alone. This sparking or priming effect of malate has been found consistently; pyruvate alone has never given an appreciable oxygen consumption. Attempts to obtain increased oxidation of malate and fumarate by the addition of DNP up to 10^{-3} M or TPN at 10^{-4} M were not successful. It was thought that the rate of malate and fumarate oxidation might be enhanced by the removal of oxalacetate, the product of the reaction. To this end glutamate was included with these two

acids to serve in a transaminating system converting oxalacetate to aspartate, but the glutamate was oxidized appreciably and there was no indication of increased oxidation of malate and fumarate. Neither acetate nor acetyl phosphate were oxidized significantly in the presence or absence of malate in several trials. The acetate-activating system was apparently absent or inactive under these conditions in the particles.

Several cofactors increase the activity of the succinoxidase system as shown in table VI. Only the omission of cytochrome c, ATP, and the substrate, succinate, caused appreciable decreases in the rates of oxidation. No appreciable endogenous respiration was ever observed with isolated particles. The inhibition by DPN indicated above was further studied and confirmed as shown in table VII. Because of the marked response to cytochrome c, it was included as a matter of course in all experiments at 0.01 mM. Doubling the concentration of cytochrome c did not increase the rate further. Although the omission of 0.001 M magnesium did not greatly affect the rate, the addition of higher concentrations has consistently enhanced succinoxidase activity (table VIII). The particles used in the experiment reported in table VIII were isolated in the presence of 0.001 M $MgCl_2$, but particles isolated in the standard manner showed similar responses. Both adenylic acid and adenosine triphosphate exert favorable effects on the rate of succinate oxidation, although it is not known whether concentrations higher than 3 mM would result in increased rates. The maximum effects of the adenylates are achieved only in the presence of relatively high concentrations of magnesium (5 mM).

The oxidation of citrate and cis-aconitate was enhanced by the presence of 0.1 mM TPN. α-Ketoglutarate oxidation was enhanced by the addition of coenzyme A. Thus, the Q_{O_2} (N) in its absence was 185 which was increased to 268 with 0.125 mg CoA per 3 ml of reaction mixture and to 313 with 0.25 mg CoA.

Coenzyme A and thiamin pyrophosphate were normally added to the reaction mixture for pyruvate oxidation. In one experiment these additives were omitted resulting in a five-fold decrease in activity from a Q_{O_2} (N) of 150 to 31. Whether both or one of these cofactors was necessary was not determined.

TABLE VII

INHIBITION OF SUCCINOXIDASE BY DPN IN PRESENCE AND ABSENCE OF ADELYLATES *

ADENYLATE ADDED	WITH 0.1 MM DPN	WITHOUT DPN
None	76	186
1 mM AMP	138	224
1 mM ATP	203	317

* Each vessel contained mitochondria equivalent to 0.58 mg of nitrogen, 17 mM PO_4 buffer pH 7.4, 0.05 mM cytochrome c, 20 mM succinate, 1 mM $MgCl_2$, 10 mM glucose, 0.35 M sucrose, in a total volume of 3.0 ml. The center well contained 0.2 ml 20% KOH.

TABLE VIII

THE EFFECT OF MAGNESIUM, FLUORIDE, AND ADENYLIC ACID ON SUCCINOXIDASE *

ADENYLIC ACID CONC	MAGNESIUM CONC	FLUORIDE CONC	Q_{O_2}(N)
0.001	0.001	.....	423
0.001	0.002	.....	477
0.001	0.003	.....	450
0.001	0.005	.....	532
0.001	0.001	0.0067	368
0.001	0.005	0.0067	423
0.003	0.001	.....	750
0.003	0.005	.....	900

* Each vessel contained mitochondria equivalent to 0.23 mg of nitrogen, 16 mM PO_4 buffer (pH 7.4), 0.01 mM cytochrome c, 20 mM succinate, 10 mM glucose, and 0.35 M sucrose in a total volume of 3.0 ml. The isolation medium contained 0.001 M $MgCl_2$ in addition to 0.5 M sucrose, 0.1 M phosphate, and 0.001 M EDTA.

A number of variations in the incubation mixture and isolation medium were made in attempts to prolong the linear rates of oxidation beyond the 25 to 35 minutes usually observed. None of these were successful, including the use of 10 mM EDTA, 1 mM $MgCl_2$, 1 mM cysteine, 1 mM BAL, 2 to 20 mM sodium fluoride, 0.1 to 0.5 mM manganese chloride or 0.05 to 0.5 mg of hexokinase in the reaction mixture.

The oxidation of succinate by these particles is inhibited by sodium malonate. In a reaction mixture containing 20 mM succinate, the Q_{O_2} (N) was 493. This was reduced to 227, 156, 70, and 38 by malonate concentrations of 0.001, 0.002, 0.005, and 0.010 M, respectively. The inhibition with the highest malonate concentration was 92%.

A number of attempts were made to obtain phosphorylation coupled to the oxidation of succinate and α-ketoglutarate by the particles. The disappearance of inorganic phosphate from the reaction medium was measured. Two experiments indicated that inclusion of 1 mM BAL in the isolation and reaction media preserved enough activity to measure a small amount of phosphate disappearance in the following mixture: 0.01 mM cytochrome C, 0.1 mM DPN, 0.1 mM ThPP, 0.1 mM TPN, 1.0 mM ATP, 0.2 mM $MnCl_2$, 1.0 mM BAL, 0.12 mg CoA, 10 mM phosphate buffer, pH 6.8, 10 mM glucose, 20 mM substrate when added, 0.5 M sucrose, 0.5 mg hexokinase, and 0.53 mg mitochondrial nitrogen in 3.0 ml. The phosphorylation was equivalent to P/O ratios (micromoles inorganic phosphate disappearing per microatoms oxygen consumed) of up to 0.47. Although phosphorylation was observed much more must be known of the effect of possible variables in the isolation procedure and reaction medium and their effect on the stability of the phosphorylating system before higher P/O ratios are obtained.

DISCUSSION

The results of this study permit the discussion of certain aspects of the physiology and metabolism

of Allomyces with respect to previous work on Allomyces, other members of the aquatic Phycomycetes, other fungi, and to classical metabolic systems.

No means was found to manipulate Allomyces into the condition commonly referred to as a "resting cell" with a low endogenous metabolism and a large increase in respiration upon the addition of respirable substrate. When the mycelium is removed from the growth medium containing the usual 0.5 % glucose, it initially respires at almost the same rate in the absence as in the presence of substrate. The endogenous respiration slowly diminished in the younger mycelia, while the older ones apparently had enough endogenous reserves to maintain near maximal rates for several hours, and then the rate diminished as in the younger mycelia. This slow decline accompanied by decrease in the ability to respond to added substrates has been noted in various fungi: *Ashbya gossypii* (30) and several species of dermatophytes (2). Other fungi may be starved by aeration in buffer, a process resulting in low endogenous rates and quite marked responses to substrates (8).

The use of a limited supply of carbohydrate, 0.05 % glucose, was a moderately successful method for growing cells which had high respiratory rates, and yet, were capable of measurable responses to external substrates. This technique has been used with Neurospora (13). Mycelium grown in this manner undergoes a more rapid decline in endogenous respiration than in capacity to respire, probably because of a more limited reserve of readily respirable material. However, with even the most favorable material, the endogenous rate is more than half the maximal rate attainable with substrate, and any attempt to assess the quantitative relationship between externally supplied substrates and gas exchange may involve a large correction for the endogenous respiration. It is possible (by growing cells labeled with C^{14}) to determine whether or not the external substrate suppresses the use of internal substances (7, 40), although with some fungi this requires an extensive series of experiments to establish the degree of suppression for each substrate used and the variation of suppression with time (3).

There are some clues to the nature of the endogenous substrate. Lynch and Calvin (25) found that a considerable amount of radioactivity was incorporated into a water-soluble polysaccharide when *Allomyces arbuscula* was suspended in a labeled bicarbonate solution—indicating that this polysaccharide was metabolically active. It may serve as an endogenous substrate. In the present experiments the respiratory quotient of 0.8 to 0.9 indicates that some substrate other than carbohydrate is quantitatively important or that there is some incomplete oxidation.

The initial steps involved in the breakdown of glucose have not been ascertained by the experiments reported here. However, the conversion of radioactive glucose into significant amounts of radioactive glucose and fructose monophosphates suggest that the pathway is phosphorylative. The recovery of labeled pentosephosphates and hexosediphosphate does not allow a differentiation between some form of a direct oxidative pathway and the Embden-Meyerhof-Parnas scheme (15). Since triosephosphate, phosphoglycerate, and phosphenolpyruvate become strongly labeled, and the inhibitors of triosephosphate dehydrogenase (iodoacetate) and of enolase (fluoride) are effective in suppressing glucose oxidation, it is probable that the Embden-Meyerhof-Parnas pathway is followed in the conversion of triosephosphate to pyruvate.

Of the many possible conversions which pyruvate may undergo, there is now evidence for several in Allomyces. Ingraham and Emerson (18) have shown that *A. arbuscula* may convert large quantities of glucose to lactic acid, but Machlis (27) has shown that, under the highly aerobic conditions of agitated culture, only a small amount of lactic acid is produced by the *A. macrogynus* used in his studies. The probable direct formation of alanine from pyruvate (14) accounts for the large quantities of alanine formed from radioactive glucose and recovered on the paper chromatograms. The third, and probably most important fate of pyruvate under aerobic conditions is its oxidation via the Krebs cycle. The evidence from studies with inhibitors, tracers, and cell-free preparations, points to the importance of this sequence of reactions. Its quantitatively important nature is demonstrated by the effect on intact cells of fluoroacetate, an inhibitor of aconitase action (31). Although the succinic dehydrogenase inhibitor, malonate, was not used in the presence of glucose with intact cells, it did depress the endogenous oxygen consumption of intact mycelia and the oxygen uptake of mitochondria to a considerable extent. The labeling of glutamic and aspartic acids also suggests that pyruvate is metabolized via the Krebs cycle.

The third type of evidence for the participation of the Krebs cycle in glucose oxidation is the ability of isolated mitochondria to oxidize the intermediates of the cycle. Mitochondria from Allomyces have activity, expressed on a nitrogen basis, of the same order as that of particulate preparations from a wide variety of higher plant tissues (12, 16) and from yeast (24). Although the oxygen consumption observed with malate and fumarate was low in comparison to that with other acids tested, malate readily served as a sparker for pyruvate oxidation, suggesting that malate was being oxidized to oxalacetate. The results of Brown and Cantino (5) are of interest in relation to these results with malate and fumarate. They found that homogenates of the closely related Blastocladiella will dismutate malate to lactate without appreciable oxygen consumption. The lactic dehydrogenase system was apparently a better electron acceptor than was oxygen under the conditions used for the assay. It is very possible that the mitochondrial preparations from Allomyces behave in the same manner.

No attempt was made to define the cofactor requirements for the oxidation of each acid, but it was

shown that TPN stimulates the oxidation of citrate and cis-aconitate and that added CoA increases the rate of α-ketoglutarate oxidation in the presence of ThPP. Furthermore, the addition of CoA and ThPP markedly enhanced pyruvate oxidation in the presence of malate. These results are consistent with the cofactor requirements of the classical system (22). The beneficial effect of adenylates upon oxidation rates of mitochondria has been observed in many systems and has been discussed by Laties (23) in terms of the function of adenylates as phosphate acceptors and as coenzymes in the transfer of esterified phosphate. The stimulation of succinoxidase by adenylic acid and ATP observed in this work might fall into either or both of these categories. A consistent effect of Mg^{++} ion in increasing oxidation rates may be involved in maintaining the integrity of the phosphorylating systems (11).

From the work reported here, the metabolism of glucose by Allomyces appears to involve the reactions of the Embden-Meyerhof-Parnas scheme from triosephosphate to pyruvate and those of the Krebs cycle from pyruvate to CO_2 and H_2O. While the mitochondria from several fungi have been shown to contain a succinoxidase system only those from yeast have previously been shown to oxidize the various intermediates of the Krebs cycle (24). It is now evident that in at least one filamentous fungus, Allomyces, there is an organized system associated with the mitochondria for carrying out these reactions.

Summary

Certain aspects of the respiratory metabolism of the aquatic Phycomycete, *Allomyces macrogynus*, were studied.

Allomyces, grown with 0.5 % glucose, was found to have an initial high rate of endogenous respiration which declined slowly with time. The effect of added substrates was to restore the oxygen consumption to the initial rate or slightly higher and to prevent the decline which took place in endogenous controls. By lowering the glucose concentration in the growth medium to 0.05 %, plants were obtained which exhibited an appreciable response to added substrates. The respiratory quotient of the endogenous respiration was found to be from 0.82 to 0.88, while that of respiration in the presence of glucose was from 1.07 to 1.22.

Glucose oxidation was inhibited by iodoacetate, sodium fluoride, sodium fluoroacetate and sodium arsenite. Acetate oxidation was not inhibited by iodoacetate.

When intact plants metabolized labeled glucose for 5 and 20 minutes, the alcoholic extracts contained mostly sugar phosphates, triosephosphate, phosphoglycerate, phosphoenol pyruvate, glutamic acid, aspartic acid, alanine, and nucleotides.

Mitochondria were isolated and found to oxidize citrate, cis-aconitate, isocitrate, α-ketoglutarate, succinate, fumarate, malate and pyruvate. The oxidation of succinate was inhibited by malonate. A slight amount of oxidative phosphorylation was observed.

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KINETICS OF GROWTH INHIBITION BY HERBICIDES[1,2]

R. E. FRANS,[3] E. F. LIND AND W. E. LOOMIS
DEPARTMENT OF BOTANY, IOWA STATE COLLEGE, AMES, IOWA

The use of herbicides has expanded phenomenally in the decade since the effectiveness of certain growth substances was first demonstrated. Research in the field, however, has been more concerned with practical problems of chemicals and rates than with considerations of physiological action. The similarity of the most used herbicide, 2,4-dichlorophenoxyacetic acid (2,4-D), to indoleacetic acid in structure and in action at low concentrations has led to the development of theories of the interference of 2,4-D with normal auxin functions (19, 21). The possibility of auxin-2,4-D relationships is strengthened by Hoffmann's work (7) showing that certain chlorinated indoleacetates were highly toxic, and that 2,4-6-trichlorophenoxyacetic acid inhibited normal auxin actions in tomato, although it was itself devoid of either auxin or herbicidal action (8).

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[3] Present address: Department of Agronomy, University of Arkansas, Fayetteville, Arkansas.

If the herbicides act upon the normal auxin sites within the cell, the more extensive literature on auxin action becomes pertinent, particularly the numerous and apparently successful attempts (5, 12, 13, 14) to characterize auxin action by the method first developed by Michaelis and Menten (15) to describe enzyme reactions. The application of these principles to growth processes has been criticized on the basis of certain techniques and assumptions (3, 9) and generally by Audus (2) who questions the advisability of attempting to relate complex growth systems to simple enzymic processes. Leopold (10), however, feels that this analysis offers the best explanation of auxin action thus far available. It is the purpose of this investigation to attempt to show that growth inhibition induced by certain herbicides and growth substances can be characterized quantitatively by a kinetic analysis of the experimental results.

MATERIALS AND METHODS

EXPERIMENTS WITH SOYBEANS: Hawkeye soybean seedlings were grown in the greenhouse in 4-inch pots

with three plants in each. Individual pots were considered to be the experimental unit. The plants were treated at the time the primary leaves were fully expanded but while the first trifoliates were still in the bud. The plants in single pots were sprayed on a rotating turntable to insure uniformity of application. All growth substances, herbicides, and mixtures of compounds were applied in aqueous solutions with 0.1 % Tergitol-7 wetting agent added to each solution. Ten milliliters of solution were applied to the plants in each pot with a paint-spray gun operating at a constant pressure of 12 psi. The pots were arranged in randomized block designs and 6 to 8 replicates were used throughout.

Growth after treatment was considered to be a function of the concentration of chemical applied and was measured as the fresh weight of all plant material above the primary leaves at 10 to 14 days after treatment. Because of inherent and uncontrollable variation between experiments the average fresh weight of plant material for each treatment was converted to percentage reduction of growth.

Experiments with Yeast: Cultures of *Saccharomyces cerevisiae* were grown in nutrient media and the growth determined turbidimetrically according to methods first described by West and Henderson (22) and later modified by Pool (16) and Swanson (17).

Nutrient agar slants were inoculated with yeast cells from the center of a pound cake of Fleischmann's bakers' yeast and incubated for 24 hours at 30° C. The slants were then stored at 5° C. Stock cultures were inoculated from the slants and incubated in 125-ml Erlenmeyer flasks for 24 hours at 30° C. Each stock culture was subcultured three times before use in the experimental cultures. The solution used for subculturing consisted of 25 ml of distilled water and 25 ml of a double strength nutrient solution made up as follows:

20 gm	glucose
3 gm	$(NH_4)_2SO_4$
2 gm	KH_2PO_4
0.25 gm	$CaCl_2 \cdot 2H_2O$
0.25 gm	$MgSO_4 \cdot 7H_2O$
2.5 gm	yeast extract (Difco)
Distilled water to make 500 ml.	

All flasks containing the nutrient media used for stock cultures and experimental cultures were plugged with cotton and autoclaved for 20 minutes at 15 lbs pressure before inoculation.

Experimental cultures of 20 ml volume containing the nutrient solution and herbicide were inoculated from stock cultures and incubated in 50-ml Erlenmeyer flasks for 15 hours. At the end of this period a reading was taken with a Klett-Summerson photoelectric colorimeter with a red (M 660) filter. Duplicate cultures were used for all treatments in these experiments. This was considered a sufficient number since the coefficients of variability for these experiments were low, ranging from about 2 to 4 %.

The assumption that the growth of the yeast was directly proportional to the concentration of cells was checked in control cultures by correlating optical density with cell counts obtained with a haemacytometer over a 24-hour period. The two measurements were found to be directly proportional to one another for a period of time well in excess of the 15 hours.

Experimental Results

Experiments with Soybeans: Soybean seedlings, when treated in the greenhouse and harvested according to the methods described, exhibited growth responses which were a function of the concentration of the growth substance or herbicide applied. The hyperbolic response of the plants to 2,4-D is shown in figure 1 A in which the experimental points represent growth inhibition as a percentage of the controls for each concentration used. It was found that this hyperbolic response curve could be described by the equation

$$c - y = \frac{x}{a + bx} \qquad (1)$$

in which $c - y$ is the percentage inhibition ($c = 100$ % growth and y is the percentage value of growth for each concentration x) and a and b are constants. This equation describes a relationship analogous to that of the initial velocity equation developed for enzyme reactions (15) and later modified to include auxin-induced growth (5, 12, 13, 14).

Preliminary attempts to transform the response curve into a straight line included a double log plot, assuming a parabolic response, and a semi-log plot, assuming that the response curve was logarithmic. The data obtained could not be expressed satisfactorily as a linear relationship by either of these methods. The hyperbolic curve described by equation *1* was transformed into a straight line, however, by plotting the reciprocal of percentage inhibition against the reciprocal of concentration. These relationships provided a mathematical basis for the development of a physiological interpretation of the data obtained.

We may postulate that a herbicide (H) will combine reversibly with some mechanism or reactive site (M) within the plant to form a complex (HM). This complex is thought to be transformed irreversibly into products which ultimately give rise to inhibition of growth, the inhibition being proportional to the concentration of such products. The above concepts may be expressed by the equation

$$H + M \underset{k_2}{\overset{k_1}{\rightleftharpoons}} HM \xrightarrow{k_3} \text{Inhibition of Growth} \qquad (2)$$

in which k_1, k_2, and k_3 are rate constants of the reactions. The general dissociation constant of the reversible reaction may be expressed as follows:

$$K_m = \frac{[H]\ [M]}{[HM]} \qquad (3)$$

in which [H], [M], and [HM] are concentrations of the herbicide, mechanism, and complex, respectively.

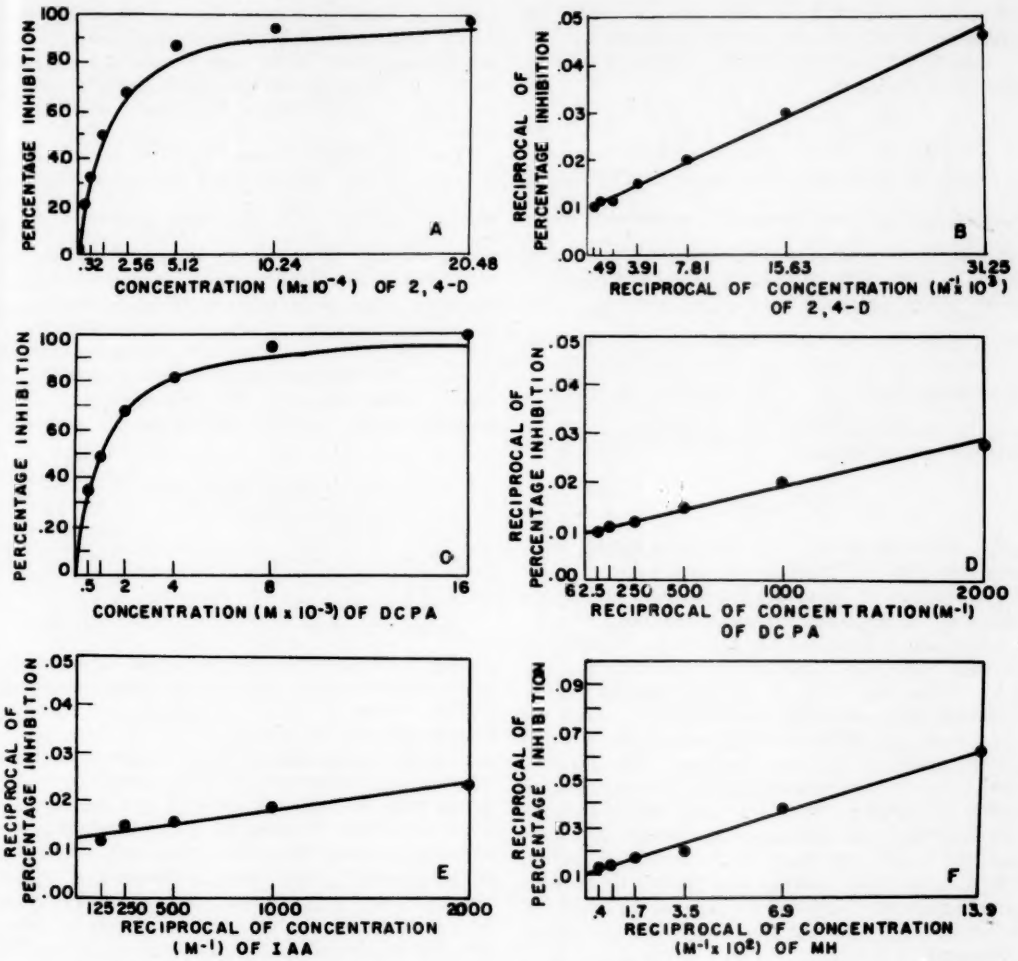

FIG. 1. Growth inhibition of soybean seedlings as a function of herbicide concentrations. Growth was measured by increase of fresh weights. A. Growth inhibition by 2,4-D. B. Reciprocal of growth inhibition by 2,4-D. C. Growth inhibition by DCPA. D. Reciprocal of growth inhibition by DCPA. E. Reciprocal of growth inhibition by IAA. F. Reciprocal of growth inhibition by MH.

The assumption made here, and which is inherent in the kinetic analysis, is that the uncoupled herbicide [H] is approximately represented by the total concentration of the herbicide. The initial velocity of the irreversible reaction is a function of the concentration of the complex and can be expressed in this manner:

$$v = k_3\ [HM] \qquad (4)$$

A quantitative measure of the hypothesis given in equation *2* may be obtained from the Michaelis-Menten equation for the initial velocity of the growth inhibition. This equation is

$$v = \frac{V_{max}\ [H]}{K_m + [H]} \qquad (5)$$

in which v is the initial velocity, [H] is the molar concentration of the herbicide, V_{max} is a constant representing the maximum velocity of the growth inhibition, attained when all the receptor sites or mechanisms are saturated with the herbicide, and K_m is the apparent equilibrium constant of the reversible reaction, or the concentration of the herbicide at which one-half the maximum velocity is attained. In the soybean experiments the measure of growth 10 to 14 days after treatment is considered to be a measure of the initial rate of growth inhibition. It is known from evidence obtained but not reported here that untreated soybeans grown and harvested under these conditions exhibit a growth rate that is essentially linear over the period used. In the yeast experiments

the measure of growth 15 hours after inoculation is considered to be a measure of the initial velocity.

Equation *5* may be re-written in the reciprocal form (11) as follows:

$$\frac{1}{v} = \frac{1}{V_{max}} + \frac{K_m}{V_{max}} \times \frac{1}{[H]} \qquad (6)$$

A plot of the reciprocal of the initial velocity (which is the same as $\frac{1}{c-y}$ in equation *1*) against the reciprocal of the concentration results in the straight line shown in figure 1 B. This straight-line plot affords a means of evaluating the constants V_{max} and K_m. The intercept of the line on the ordinate is $\frac{1}{V_{max}}$ and the slope of the line is $\frac{K_m}{V_{max}}$. The value for the intercept may be obtained directly from the graph and substituted in the expression

$$\frac{K_m}{V_{max}} = \text{slope} \qquad (7)$$

to get the value for K_m. In these experiments, however, the line was fitted to the experimental points by calculation of linear regression, from which the values of the slope and intercept were obtained.

Equation *5* expresses the hyperbolic relationship between percentage inhibition and concentration (fig 1 A). When the constants, derived from the double reciprocal plots, are inserted in equation *5*, values for the percentage inhibition for each concentration of the growth substance can be calculated. These values were found to correspond closely to the experimental values as shown in figure 1 A where the continuous line represents the calculated percentage inhibition and the points represent the experimental values. The data for 2,4-D indicate that the kinetic analysis fits the response of soybean seedlings to the inhibiting action of 2,4-D over the range of concentrations investigated.

The results from experiments with 2,2-dichloropropionic acid (DCPA), indole-3-acetic acid (IAA), and maleic hydrazide (MH), were also subjected to the kinetic analysis. The hyperbolic response curve and double reciprocal plots for DCPA are shown in figures 1 C and 1 D. Results obtained with this herbicide were very similar to those obtained with 2,4-D. A good fit of the reciprocal plot of the data to a straight line was obtained (fig 1 D), and the calculated and experimental percentage inhibition showed close agreement (fig 1 C). It was found that higher concentrations of IAA than of 2,4-D were needed to inhibit growth to the same extent (fig 1 E). Furthermore, the maximum rate of inhibition obtained with IAA was 81 per cent, compared with 100 % with 2,4-D and DCPA. Higher concentrations of maleic hydrazide than of 2,4-D or DCPA were needed to cover the inhibitory range (fig 1 F). The maximum rate of inhibition of 100 % was reached with the highest concentration used. Results with other herbicides analyzed by these procedures, but not reported here, include 2,4,5-trichlorophenoxyacetic acid (2,4,5-T), trichloroacetic acid (TCA), and 3-amino-1,2,4-triazole (AT). It was found that the kinetic analysis could be successfully applied to the data from these experiments.

The value obtained from K_m is the concentration at which one-half the maximum rate of inhibition is attained $\left(\frac{V_{max}}{2}\right)$. The K_m value also serves as a measure of the affinity of the compound for the reactive site or mechanism within the plant, and may, therefore, serve as an index or means of expressing the relative toxicities of growth substances or herbicides. The smaller the value of K_m, the greater is the affinity. The compounds may be ranked according to the K_m values obtained. The following array is arranged in order of decreasing affinities for soybeans:

	K_m
2,4,5-Trichlorophenoxyacetic acid	6.22×10^{-6}
2,4-Dichlorophenoxyacetic acid	1.18×10^{-4}
Indole-3-acetic acid	5.04×10^{-4}
3-Amino-1,2,4-triazole	6.94×10^{-4}
2,2-Dichloropropionic acid	9.28×10^{-4}
Trichloroacetic acid	1.00×10^{-3}
Maleic hydrazide	3.79×10^{-3}

It may be of significance in speculating on the nature of the receptive site or sites, that the three compounds ranking highest according to their affinities are those known to function as auxins.

This kinetic analysis of growth inhibition affords a means of evaluating quantitatively the response obtained when the inhibition induced by a primary, active compound is modified by the presence of a secondary compound, which may or may not be active in producing growth inhibition. In the simplest type of interaction two such compounds may be specific in their requirements for the same mechanism or receptive site in the plant. In this type of action, the molecules of the two compounds compete with one another for these sites. If such is the case, the double reciprocal plot (11) will result in a line whose slope is increased as compared with that representing the primary compound alone, but the intercept remains unchanged.

An example of this type of response is shown in figure 2 A. Various concentrations of 2,4-D and 2,4,5-T were applied singly and in combination to soybean seedlings. The primary compound was considered to be 2,4-D and 2,4,5-T the competitor. The reciprocal of percentage inhibition is plotted in figure 2 A against the reciprocal of the concentration of 2,4-D for each of the four levels of 2,4,5-T. To avoid confusion, only two of the four lines are shown in the graph. The lower line is the double reciprocal plot of 2,4-D with no 2,4,5-T added. The slopes of the other three lines were all greater, an indication that measurable competition occurred between 2,4-D and 2,4,5-T. It should be emphasized that as the slopes of the lines in these graphs increase, the amount of growth inhi-

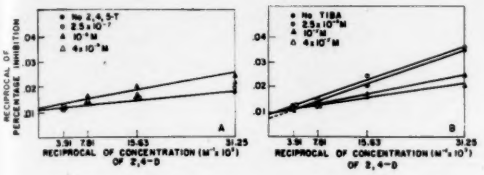

FIG. 2. The reciprocal of growth inhibition of soybean seedlings as a function of the reciprocal of the concentration of 2,4-D at four levels of (A) 2,4,5-T and (B) TIBA.

bition decreases. The more shallow and flat the slope of the line, the greater is the degree of plant growth inhibition.

A common V_{max} is assumed for the four reactions depicted in figure 2 A, although small differences were encountered among the intercepts on the ordinate. Calculated *t*-values showed that differences between slopes of the lines representing the intermediate and high levels of 2,4,5-T and the zero level of 2,4,5-T were significantly greater at the 5 and 10 % level respectively. Although the upper line in the graph represents the intermediate concentration, no significant difference existed between the slope of this line and the slope of the line representing the high concentration of 2,4,5-T. There was no apparent difference between the slopes of the line representing the low concentration of 2,4,5-T (2.5×10^{-7} M) and the line representing 2,4-D only.

Another type of response is shown in the effect of mixtures of 2,3,5-triiodobenzoic acid (TIBA) and 2,4-D on soybeans (fig 2 B). TIBA has been shown to interact with auxin at low concentrations to increase the stimulation of growth (1, 18), but to act as an antiauxin at high concentrations (1, 6, 20). In this experiment TIBA alone inhibited growth and, in combinations, enhanced the action of 2,4-D. This effect is shown in the double reciprocal plots by the decrease in the slopes of the lines below that of the line representing the action of 2,4-D alone. The slopes of the lines representing the intermediate and high levels of TIBA were significantly less than the slope of the line representing the zero level of TIBA at the 5 % level. The lines for the two high levels of TIBA differed significantly at the 10 % level. The intercepts of the lines on the ordinate are again assumed to be nearly identical.

EXPERIMENTS WITH YEAST: Measurements of growth made in experiments with yeast were analyzed in the same manner as those of the soybean experiments. It was generally found that the concentration of chemical needed to obtain a given response in yeast was considerably higher than that for soybeans. Also, a narrower range of concentrations covered the complete range of growth inhibition.

The double reciprocal plots for IAA and 2,4-D are shown in figures 3 A and B. The range of concentrations used in these two experiments was identical. The maximum rate of inhibition attained with IAA was less, however, being of the order of 70 % compared to 100 % with 2,4-D. This is in agreement with results obtained in the soybean experiments. It will be noted in the 2,4-D experiment (fig 3 B) that the line does not intercept the ordinate at 0.01 (the reciprocal of 100 % inhibition). The calculated values for V_{max} and K_m, therefore, have no physiological interpretation since the maximum inhibition can be no greater than 100 %. A re-check of the growth of yeast in solutions containing 2,4-D indicated that the size as well as the number of cells was reduced at high concentrations. This effect may have caused the irregularities in the values obtained for V_{max} and K_m.

Mixtures of TIBA and 2,4-D increased inhibition of yeast growth over that observed with 2,4-D alone (table I and fig 3 C). This action was similar to that observed in soybeans. The slope of the line representing the high concentration of TIBA was signifi-

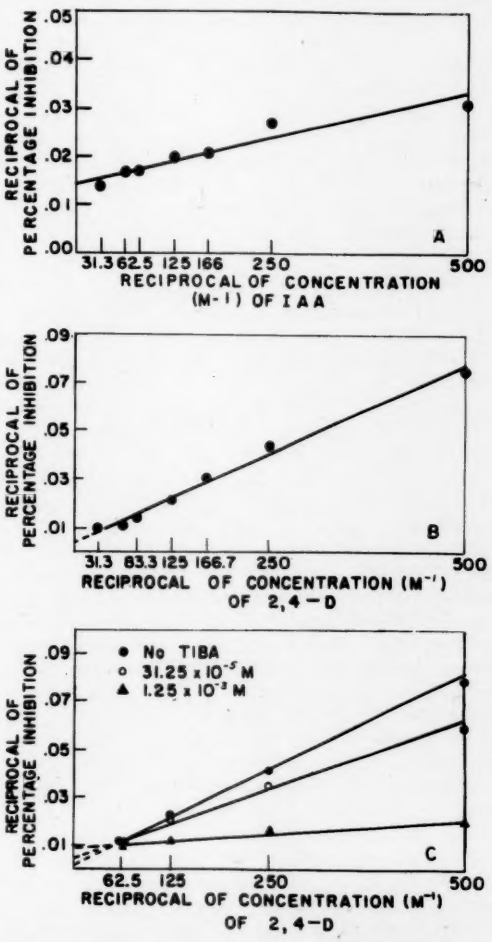

FIG. 3. The reciprocal of yeast growth inhibition as a function of the reciprocal of concentration of (A) IAA, (B) 2,4-D, and (C) 2,4-D at three levels of TIBA.

TABLE I

PERCENTAGE INHIBITION OF YEAST GROWTH AS A FUNCTION OF CONCENTRATIONS OF 2,4-D AND TIBA ADDED TO CULTURE MEDIA SINGLY AND IN COMBINATION

CONC (M) OF 2,4-D	CONC (M) OF TIBA		
	NONE	3.1×10^{-4}	1.3×10^{-3}
None	...	2.3	15.4
2×10^{-3}	12.7	16.7	49.3
4×10^{-3}	24.4	29.0	62.2
8×10^{-3}	44.8	46.2	85.1
1.6×10^{-2}	82.4	85.4	99.6

cantly less (5 % level) than that of the line representing the zero concentration. At the low concentration of TIBA, however, the difference between slopes was significant only at the 20 % level. This was probably due to the fact that TIBA alone, at this concentration (3.1×10^{-4} M), inhibited growth by only 2.3 % (table I). It will be noted that the increase in growth inhibition at this concentration is 3 to 4 % over that shown by 2,4-D alone, an effect which again may be considered additive. With a high concentration of TIBA (1.3×10^{-3} M) and low or intermediate concentrations of 2,4-D, however, inhibition of growth was increased by 35 to 40 %. This apparent synergistic action of TIBA has been noted in other studies on auxin interactions (1).

DISCUSSION

The kinetic analysis of sucrase action developed by Michaelis and Menten (15) has been shown to apply generally to enzyme reactions. Recently, similar analyses have been applied successfully to growth stimulation by auxins (5, 12, 13, 14). Our results indicate that growth inhibition by auxin and other herbicides can be described by a similar analysis.

The following hypothesis was formed: 1) Plant growth inhibition caused by growth substances or herbicides originates at the molecular level. 2) The herbicide combines reversibly with a mechanism or site within the plant. 3) The herbicide-mechanism complex reacts irreversibly to form products which cause growth inhibition in direct proportion to the concentration of such products. The derivation of the mathematical expressions of the hypothesis depends upon the reversibility of the reaction between the herbicide and the mechanism in the plant, and also upon the fact that the breakdown of the complex is probably an irreversible, first order reaction.

This method of analysis provides a physiological basis for interpretation of inhibitory action by herbicides and also incorporates the desirable feature of permitting a linear plot of the transformed data points. Blackman (4) obtained linearity by transformation of data points to "probits," but his analysis cannot be readily interpreted in physiological terms. A kinetic analysis, however, will provide a practical means of evaluating quantitatively the biological activity of auxins and herbicides.

It has been shown that the results obtained in soybean experiments with single chemicals can be interpreted in the light of the kinetic analysis employed. The successful application of these principles to growth inhibition responses does not provide proof of the mechanism with which the growth substance or herbicide combines. It seems probable, however, that the synthetic auxins (2,4,5-T, 2,4-D and IAA) will combine with the mechanism or sites necessary to auxin-induced growth. Since our hypothesis assumes that this first reaction has little or no action in inhibiting growth, the greater toxicity of 2,4-D compared to IAA, for example, would be explained on the basis of a more rapid reaction in the second, irreversible step (k_3 of equations *2* and *4*). Hoffmann's findings (7) that certain chlorinated derivatives of IAA were highly inhibitive of growth tends to support this hypothesis.

Presumably the action of non-auxin-like inhibitors (TCA, MH, AT, DCPA) follows a different course. These compounds do not possess the structural requirements which have been shown to be necessary for auxin action. It is probable, therefore, that these agents function by means of a direct combination with secondary sites, or interference with substances required for growth. Native auxin would still react normally with the stimulatory site. Growth inhibition induced by the applied chemical, however, could mask or outweigh growth stimulation since the inhibitor is present in relatively large quantities.

It is apparent, from the concentrations used, that yeast is more tolerant to 2,4-D and IAA than soybeans. This tolerance is evidenced by the relatively steep slopes of the double reciprocal plots. This seeming inefficiency of the herbicide in arriving at and combining with a particular site may exist because part of the herbicide is being tied up or broken down before it could combine with the receptor. These products may possibly be metabolized by the organism up to certain levels of saturation, after which intact molecules of the herbicide or growth substance are free to react with the receptor entity. Such an interpretation might explain why the yeast organism, even though it is continually bathed in a solution containing the growth substance for the entire growth period, requires much higher concentrations for inhibition than do soybeans.

Data obtained from the soybean experiments with mixtures of herbicides indicate that 2,4,5-T competitively inhibited the action of 2,4-D at low concentrations of the latter. It is probable that both of these compounds are specific in their action for the same site or mechanism leading to growth inhibition. It is also likely that these sites are closely related to, if not the same, sites active in auxin-induced growth. Although a secondary reaction has been visualized above for the completion of growth inhibition effects, it is most likely that the competitive action between these synthetic auxins occurs at the primary site, leaving a limited number of molecules free to react with the secondary or inhibitory site or mechanism.

The action of TIBA in increasing the toxicity of 2,4-D, particularly on yeast (table I and fig 3 C), appears significant. On the hypothesis that TIBA is a competitive antiauxin (1, 6, 20), we might assume that the reduced number of sites available for the first, reversible reaction of 2,4-D accelerates the second inhibiting reaction. A second possibility, that the inhibiting action of 2,4-D is due to an interference with auxin at the primary site, seems less probable. TIBA was relatively non-toxic when used alone on yeast or when used with 2,4-D at lower concentrations (table I). At moderate concentrations, however, where the saturation of primary sites should have been high, the effect of TIBA with 2,4-D was striking. Compare, also, figure 2 B.

Growth inhibition, therefore, seems to lend itself to the physiological interpretation of this analysis, which in these studies, afforded the simplest such explanation possible under the experimental conditions employed. It is acknowledged that more critical studies of growth inhibition will need to answer many vital questions, such as the effect of pH upon plant response, the possibility of reversing growth inhibition upon removal of the inhibitor, and the possibility of further mathematical refinement of the analysis. The importance of these problems cannot be minimized. It is believed, however, that the evidence presented here, that inhibition proceeds on the molecular level and can be satisfactorily characterized by a kinetic analysis, offers a foundation for further investigations into the physiological meaning of plant growth inhibition.

Summary

Several herbicides and growth substances were applied singly and in combinations at varying concentrations to soybean seedlings and yeast cultures in an attempt to characterize accurately the growth inhibition responses obtained. The growth measurements were subjected to a kinetic analysis in analogy with that proposed for enzyme systems and later modified to include auxin-induced growth.

Growth inhibition of soybean seedlings induced by single applications of 2,4-D, DCPA, 2,4,5-T, TCA, AT, IAA, and MH, satisfactorily supported the hypothesis inherent in the kinetic analysis. It is suggested that calculated K_m values may be of value in determining relative affinities of these compounds for sites or mechanisms within the plant leading to growth inhibition.

Results obtained by single additions of 2,4-D and IAA to yeast cultures showed satisfactory agreement to straight line reciprocal plots and between calculated and experimental percentage inhibition.

Growth inhibition of soybean seedlings induced by low concentrations of 2,4-D was competitively inhibited by 2,4,5-T. The competitive action was not evident at higher concentrations of the 2,4-D. The effect of TIBA was to increase additively the inhibition caused in soybeans by 2,4-D, and synergistically the inhibition caused in yeast by 2,4-D.

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THE SUBSTITUTION OF COMPLEXING SUBSTANCES FOR BORON IN PLANT GROWTH[1,2]

JOHN SKOK

DIVISION OF BIOLOGICAL AND MEDICAL RESEARCH, ARGONNE NATIONAL LABORATORY, LEMONT, ILLINOIS

The essentiality of the micronutrient boron is well established, although our knowledge concerning its exact function is still largely empirical. Consideration has been given to the possible existence of a relationship between its biological role in the plant and the capacity of the borate ion to complex with various polyhydroxy and related compounds including several of the common sugars (see reviews by Gauch and Dugger (2) and Zittle (14)). Hoagland (3) and Winfield (13) were among the first to call attention to the possibility of such a relationship, and during the past few years experimental work has been directed to determine its nature (1, 5, 8, 10, 11). Gauch and co-workers (1, 5, 8) in particular have studied this problem; they favor the hypothesis that a major function of boron is in sugar translocation by the formation of a sugar-borate complex which passes more readily through membranes. Scripture and McHargue (7) have also considered boron as functional in translocation of carbohydrates, but not in light of the formation of a complex. Recently McIlrath and Palser (4, 6) have studied physiological and anatomical plant responses to boron with special reference to carbohydrate relationships.

Since various other elements such as strontium, aluminum, and germanium will also form complexes similar to the borate complexes, it was decided to determine whether certain of these elements may substitute partially for boron in the growth of plants. If boron deficiency symptoms are partially alleviated by the addition of any of these other elements in the absence of boron, this would constitute evidence that the essentiality of boron for plant growth is probably in part related to the complexing property of borate. This report presents in full the results of experiments which were summarized previously (10, 11).

MATERIALS AND METHODS

Mammoth Russian sunflower was used in all experiments. The plants were grown either in quartz sand in the greenhouse or in nutrient solutions in a growth room providing a controlled environment.

Experiments 1 and 4 were carried out in the greenhouse during the month of October under unsupplemented natural light conditions. Seed were sown in sand and 10 days later the seedlings were transplanted to individual 1.5-liter glazed porcelain crocks containing sand. The plants were supplied daily with nutrient solutions of the following composition: $Ca(NO_3)_2$, 0.0045 M; KH_2PO_4, 0.0025 M; $MgSO_4$, 0.0015 M; Fe, 5 ppm, supplied as the chelated ferric complex, Sequestrene Na Fe (monosodium ferri-ethylene-diamine-tetraacetate dihydrate); Mn, 0.5 ppm; Zn, 0.5 ppm; Cu, 0.125 ppm; and B, 0.5 ppm or as otherwise stated. Stock solutions were made up in boron-free glassware.

All of the other experiments were carried out in a growth room under constant temperature and light conditions. The light was supplied by suspended panels containing both 72-inch T 8 Standard Cool white fluorescent tubes and 75-watt reflector-type filament lamps delivering approximately 1500 ft-c at the leaf surfaces. A daily photoperiod of 16 hours, a temperature of 75° ± 2° F and a relative humidity of 50 % ± 15 % were maintained. The seed were sown in quartz sand and watered with tap water, and the plants were used one week after sowing; at this time the seedlings had expanded cotyledons, but no emerged leaves. They were transferred to nutrient solutions contained in wide-mouthed, low-boron, soft glass, quart fruit jars wrapped in black cloth to exclude light. The plants were supported either by cork pads or by slotted stainless steel holders and held in place by a pliable asbestos type mastic (Kalk-Kord). The slot in the stainless steel holder permitted removal of plants without injury. The solutions were not aerated, since preliminary experiments indicated that growth was not improved by aeration. Under these culture conditions, boron deficiency symptoms appeared considerably earlier than under the greenhouse sand-culture conditions.

Uniformly labeled C-14-sucrose was used to study sugar translocation. Plant tissues were assayed after oven drying at 80° C. Ground samples were counted at infinite thickness in a 2.5-cm² cup using a thin end window G.M. counting tube. Relative count rates can be converted to absolute activities and actual amounts of sucrose by comparison of count rate of sample to the count rate of a standard sample previously assayed for absolute activity after combustion (9).

All measurements and assays were analyzed by the use of the *t*-test to determine whether differences between experimental treatments were significant.

EXPERIMENTATION AND RESULTS

EXPERIMENT 1: The first substance to be tested was strontium as $SrCl_2$. This experiment was conducted in the greenhouse and the plants were grown in quartz sand and given daily applications of nutrients. There were ten plants per treatment with the following treatments included: 1) 0.5 ppm B added; 2) no B added; 3) no B but 0.5 ppm Sr added; 4) no B but 1.5 ppm Sr added. The plants were grown for 20 days at which time half of them (5 plants of each treatment group) were harvested.

[1] Received January 3, 1957.

[2] Work performed under the auspices of the U. S. Atomic Energy Commission.

TABLE I

EFFECT OF STRONTIUM ON GROWTH OF SUNFLOWER PLANTS IN ABSENCE OF BORON
(AVERAGE OF 5 PLANTS)

TREATMENT	HT FROM COTYLEDONARY NODE TO TIP	DRY WT TOPS
	cm	*gm*
+B (0.5 ppm)	25.1 ± 0.8 **	3.18 ± 0.14 **
−B	13.1 ± 0.9	1.66 ± 0.14
−B, +Sr (0.5 ppm)	17.4 ± 1.5 *	2.12 ± 0.16
−B, +Sr (1.5 ppm)	17.7 ± 1.4 *	2.25 ± 0.13 *

Significant increase over "−B" indicated by * at 5% level and by ** at 1% level.

Boron deficiency symptoms had become evident in the −B plants by about 12 days; these individuals were considerably stunted as compared with those receiving boron. The −B plants receiving both levels of Sr were also stunted and had smaller leaves than the +B plants, but their growing tips had not developed necrotic areas as was the case with the plants receiving neither B nor Sr. Several days later the −B plants receiving Sr also began to develop necrotic tips, but the severe and characteristic B deficiency symptoms had been clearly delayed for about 5 days by the addition of Sr. Height measurements and total dry weights of the top portions of the plants (table I) indicate that Sr had increased growth in the absence of B. Substitution of Sr for B, however, was very temporary.

The remaining half of the original plants were maintained for an additional week. During this time all plants which had received no B for the preceding 20 days were given 0.01 ppm B as well as the scheduled amounts of Sr. Those previously receiving 0.5 ppm B continued to receive the same amount. The results are shown in table II. More growth, as measured by dry weight accumulation, was made by −B plants receiving 1.5 ppm Sr than similar plants receiving no Sr, when addition of minimal amounts of B was made available for seven additional days.

TABLE II

EFFECT OF STRONTIUM AND MINIMAL AMOUNTS OF BORON ON GROWTH OF BORON-DEFICIENT SUNFLOWER PLANTS
(AVERAGE OF 5 PLANTS)

TREATMENT		DRY WT TOPS
FIRST 20 DAYS	FOLLOWING 7 DAYS	
		gm
+B (0.5 ppm)	+B (0.5 ppm)	9.76 ± 0.92 **
−B	+B (0.01 ppm)	2.31 ± 0.27
−B	+B (0.01 ppm)	.....
+Sr (0.5 ppm)	+Sr (0.5 ppm)	3.12 ± 0.24
−B	+B (0.01 ppm)	.....
+Sr (1.5 ppm)	+Sr (1.5 ppm)	3.45 ± 0.38 *

Significant increase over "−B for 20 days, followed by 0.01 ppm B for 7 days" indicated by * at 5% level and ** at 1% level.

EXPERIMENT 2: The second complexing substance tested was germanium as germanium dioxide (germanic acid, GeO_2, soluble form, sp. gr. 4.7). The plants were grown in nutrient solutions in the controlled environment room under the conditions already described and the nutrient solutions were made up with boron (+B), without boron (−B), and without boron but with germanium (−B, +Ge). Boric acid and germanic acid were present at concentrations of 5×10^{-5} M (the approximate molar concentration of boric acid when B is supplied at 0.5 ppm).

Of all the complexing substances tested, Ge gave the most striking results in the alleviation of boron deficiency symptoms. The −B plants developed noticeable symptoms by the fourth day from time of transference to solutions and easily discernible symptoms (discolored roots, wrinkled, light, and discolored leaves, and bronzed growing tips) by the fifth day,

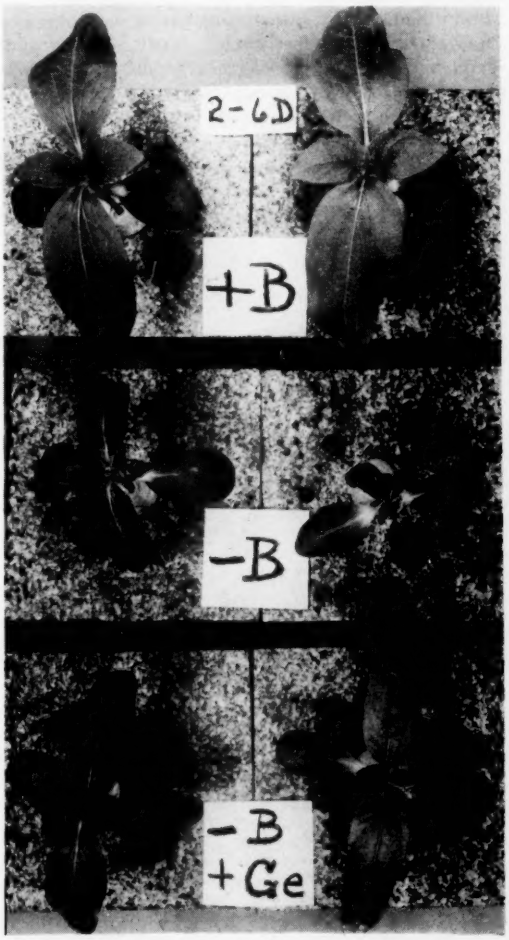

FIG. 1. Top view of sunflower plants showing terminal regions. Sixth day of growth in their respective solutions. Treatments indicated by labels.

while the – B, + Ge plants showed none of these symptoms. On the 6th day necrotic tips were present in the – B plants but not in the – B, + Ge plants; however, the – B, + Ge plants were slightly smaller than the + B plants (fig 1). The roots of the – B, + Ge plants were less well developed than those of the + B plants; they were yellowish in color, more branched than the controls, with stubby terminals, whereas the roots of the + B plants were white and fibrous. The roots of the – B plants were poorly developed, grayish in color, and less fibrous than those of the + B plants, but they lacked the stubby, bulbous form of the root extremities exhibited by the – B, + Ge plants. At the 9th day, the – B, + Ge plants were still free from pronounced symptoms, but some plants were just beginning to develop slightly buckled leaves and discolored tips (figs 2 and 3). The – B plants at this time showed very severe symptoms; all plants had dead tips and in some cases the entire top portion appeared dead. Similar and reproducible results were obtained in four different experiments, clearly showing that germanium will delay severe boron deficiency symptoms for 5 to 6 days under the present experimental conditions, although it cannot replace boron indefinitely. Increases in height of – B, + Ge plants over – B plants are not striking, but the increases in total

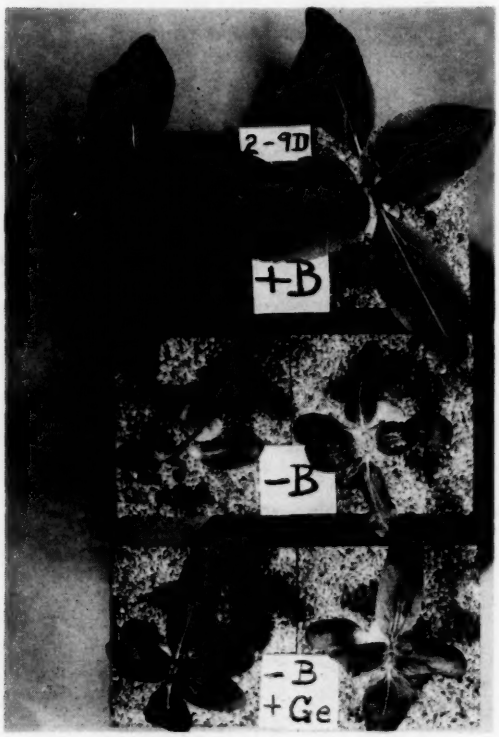

FIG. 2. Top view of sunflower plants showing terminal regions. Ninth day of growth in their respective solutions. Treatments indicated by labels.

FIG. 3. Side view of sunflower plants showing relative size. Ninth day of growth in their respective solutions. Treatments indicated by labels.

dry weight accumulation at the 9th day (table III) indicate that more growth is made if germanium is available during the absence of boron. Germanic acid samples were submitted to the spectrographic laboratory of our Chemistry Division and were found to be free of detectable boron.

An experiment was done to determine the effect of Ge on growth in the presence of B. The top portions of plant receiving both boron and Ge resembled the + B controls, but their roots were stubby. Thus it appears that this characteristic root development is initiated by Ge rather than related to B deficiency. It is interesting to find that, although Ge can temporarily substitute for boron in the maintenance of functional top development, its presence has one effect similar to that of B deficiency, that is, it can prevent the formation of a fibrous root system.

EXPERIMENT 3: Since aluminum is also a complexing substance, it was tested as a replacement for B in the form of $AlCl_3$, 5×10^{-5} M. The plants were grown under the conditions described in experiment 2. Plants receiving aluminum but no boron grew taller than the – B plants receiving no Al, but differences in dry weight accumulation were not significant. The development of B deficiency symptoms progressed about equally in the two groups.

Plants receiving 0.0005 ppm B (a total of 0.5 μgm B in the solution available to each plant) do not show typical boron deficiency symptoms until about three days later than similar plants receiving no added B; they are, however, stunted in comparison to plants grown with 0.5 ppm B. If either Al, Sr, or Ge is added (at 5×10^{-5} M) along with 0.0005 ppm B, the plants develop B deficiency symptoms later, and are less stunted, than plants receiving either 0.0005 ppm B or one of the substituted elements alone. This suggests that B and some of the other complexing substances may function in certain common ways to a limited extent even though none of the latter can replace B entirely.

EXPERIMENT 4: The foregoing experiments suggest that the complexing property of the borate ion may be involved in some physiological function of

TABLE III

EFFECT OF GERMANIUM ON GROWTH OF SUNFLOWER PLANTS IN ABSENCE OF BORON

TREATMENT	DRY WT ENTIRE PLANT (AV OF 15 PLANTS)
	mg
+B	406.5 ± 15.3 **
−B	164.6 ± 9.0
−B, +Ge	217.7 ± 11.2 **

** Difference from "−B" lot significant at 1 % level.

boron. However, they do not illustrate the manner in which the complexing reaction functions.

If B is essential for sugar translocation and B deficiency symptoms result from a shortage of carbohydrate material, it would appear that application of such material to these deficient sites should at least in part replace the boron requirement. This possibility was tested by utilizing + B and − B plants grown under conditions similar to those of experiment 1. The − B plants were divided into three groups of 10 plants each; plants of group 1 received no other treatment; plants of group 2 were sprayed daily with 1 % sugar solution made up of equal amounts of sucrose and dextrose; plants of group 3 were sprayed daily with a 0.1 % citric acid solution. The spray material was applied to the entire plant but particular precautions were taken to provide good coverage on the apical bud region. Citric acid was included because organic acids are generally more penetrating than sugars.

This experiment was carried on for a period of 3 weeks during which daily observations were made on the development of deficiency symptoms. Neither the sugar nor the citric acid spray delayed or alleviated the B deficiency symptoms, nor did they increase growth by measurable increases in height or accumulation of dry matter. Sisler et al (8) were also unable to increase growth by applying sugar to tips of boron-deficient plants. McIlrath and Palser (4) detected no, or very slight, increases in growth of B-deficient tomato, turnip and cotton plants after spraying with sugar, and the boron deficiency symptoms were not alleviated by the treatment. If B functions primarily in the translocation of sugar to the normally actively growing tips, the application of carbohydrates to the tips of B-deficient plants should have permitted some measurable increase in growth and some alleviation of visible boron deficiency symptoms.

EXPERIMENT 5: The results of experiment 4 do not support the thesis that the terminal regions of B-deficient plants become necrotic because of a failure of sugar translocation from the leaves to these regions. Another experiment using C-14-labeled sucrose was carried out to measure more directly the effect of boron on translocation. Since germanium gave temporary but definite alleviation of boron deficiency symptoms, its effect on sugar translocation was also tested.

Young sunflower seedlings were grown in sand in the controlled environment room, supplied only with tap water. They were used in the experiment the 9th day from sowing of the seed at which time they consisted of axes, expanded cotyledons and expanded first pairs of leaves. Eighteen uniform plants were selected and divided into three lots of six plants each. C-14 sucrose, uniformly labeled at a level of 236 μc/gm, was used to prepare three solutions (all 10 % sucrose) as follows: 1) sugar only, 2) sugar + B, and 3) sugar + Ge. Each lot of plants received applications of one of the three sugar preparations. The sugar solution was administered by placing 50 μl of a solution on each cotyledon inside a lanolin ring, thus supplying each plant with 100 μl solution which contained 10 mg sugar and 2.36 μc of C-14 activity. The sugar + B solution contained 40 μgm B in each 100 μl aliquot and the sugar + Ge solution contained an equimolar amount of GeO_2 (268 μgm Ge per 100 μl).

Ninety-two hours later, the plants were harvested; the cotyledons were excised and discarded and the remainder of each plant was divided into epicotyl and hypocotyl portions and assayed for radioactivity. The amount of sugar that was translocated from the site of application to other portions of the plant was found to be extremely variable within treatments, and no significant differences were found to exist between the three treatments of this experiment. Later experiments have likewise failed to show any direct enhancement of translocation by B, although indirect effects can undoubtedly be expected.

DISCUSSION

The temporary alleviation of B deficiency symptoms by complexing elements as Sr, Al and particularly by Ge, strongly suggests that the physiological role of B is in part related to the complexing property of the borate ion, but the basis of this relationship has not been revealed. The fact that boron is not reutilizable, that is, that an available supply is required at all times, suggests that this complexing reaction may be related to the formation of a structural unit or "building block" rather than to a metabolic step reaction.

The results of these experiments do not support the hypothesis that translocation of sugar is enhanced by the formation of a sugar-borate complex. Recent experiments (12), to be described more fully in the future, show a close relationship between B and cellular activity at the meristems and suggest that the apparent effect of B on translocation is related to such cellular activity rather than to the formation of a B-sugar complex.

SUMMARY

Several complex-forming elements including strontium, aluminum, and, particularly, germanium, were found to alleviate boron-deficiency symptoms temporarily and to increase growth of sunflower seedlings significantly in the absence of boron. These results

provide evidence that the physiological role of boron is likely related in part to the complexing property of the borate ion. On the basis of translocation studies utilizing C-14 sucrose and tests on the effect of sugar and citric acid applications to boron-deficient plants, it does not appear that boron is directly functional in sugar translocation because of the formation of a sugar-borate complex.

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THE EFFECT OF CALCIUM ON POTASSIUM ACCUMULATION IN CORN AND SOYBEAN ROOTS[1]

J. S. KAHN and J. B. HANSON

Department of Agronomy, University of Illinois, Urbana, Illinois

It has often been observed that different species of plants will accumulate markedly different amounts of nutrient cations from the same root medium (3). This is particularly striking in the case of mono- and divalent cation absorption by grasses and legumes (5, 8); the former tend to accumulate much more potassium and less calcium than the latter.

Such differential absorption appears to have ecological and agronomic importance when grass and legumes species are members of the same plant society, as in mixed pastures. The productiveness of legumes in such pastures tends to fall sharply after a few years, a process which can be slowed down, but not stopped, by liberal applications of potassium fertilizer (1). Apparently, the potassium accumulating mechanism of legumes cannot compete successfully with that of grasses unless the concentration of the ion is quite high.

The amount of K that a plant absorbs, however, will depend not only on the species and the concentration of the ion in the root medium, but also on the relative amounts of Ca and Mg present. It has been demonstrated that the total amount of cations absorbed by a plant from differing media tends to approach a constant, but that the relative accumulation of K, Ca, and Mg is dependent upon the ratios of these ions in the nutrient medium (4, 14). High concentrations of K will tend to depress the accumulation of divalent cations, and vice versa. Olsen (11) has demonstrated that different species respond differently to changing Ca/K ratios, but the species tested did not include legumes.

A complicating factor of unknown significance in the potassium accumulation of plants is the promoting effect of Ca, and to a lesser degree of Mg and other di- and trivalent ions, on the uptake of K and associated anions (the Viets effect) (16). Studies of this effect have been made with barley (12) and Mung bean roots (15). However, no direct comparison of

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the magnitude of this effect in grass and legume species has been made and no assessment as to the importance of this effect in the relative cation composition of the two families is possible.

The experiments described here were performed in order to make such a comparison and to evaluate the effect of Ca on K uptake in terms of the kinetic treatment of Epstein and Hagen (6).

Materials and Methods

Hybrid corn (WF 9 × M 14) and soybeans (Hawkeye) were used as representative grass and legume species. A preliminary nutrient culture experiment was run in the greenhouse to make certain these plants were characteristic of their families with respect to K and Ca accumulation.

Five-day-old seedlings were fixed with cotton plugs in a masonite lid, 2 corn and 2 soybean seedlings per lid, and the roots suspended in 7 liters of aerated, modified Hoagland solution in a glazed earthenware crock. By varying the relative amounts of $Ca(NO_3)_2$ and KNO_3 used, the Ca/K ratios indicated in figure 1 were obtained, all other ions remaining constant. The pH varied from 5.6 to 6.1; solutions were changed every 6 days. The experiments were done with full and with 1/5 strength nutrient solutions.

The plants were harvested after 4 weeks, weighed, dried, reweighed and ground in a Wiley mill. Samples were wet-ashed with nitric and perchloric acids for analysis. K and Ca were determined in a Perkin-Elmer flame photometer, Ca + Mg with Versenate (2) and Mg calculated by difference. The Ca standard solutions contained the average amount of K and Mg found in the samples as interfering ion.

Excised root experiments were done with the entire primary root from 4-day-old seedlings. The seeds were germinated at 28° C in Pyrex baking dishes on a 0.5-inch (1.25-cm) bed of washed quartz gravel covered with paper toweling and wetted with 10^{-4} M $CaCl_2$. The humidity in the germinator was maintained near saturation. Growth was vigorous, and root hairs and secondary roots were present.

Four entire primary roots were cut into approximately 1-inch (2.5-cm) pieces and placed in a 125-ml Erlenmeyer flask with 25 ml of solution; each treatment was replicated three or four times. Potassium solutions were labelled with Rb^{86}, and equimolar concentrations of Cl and PO_4 were present as anion; pH varied from 5.5 to 5.8. Solutions contained 1 % sucrose as it was found to decrease variability between replicate flasks.

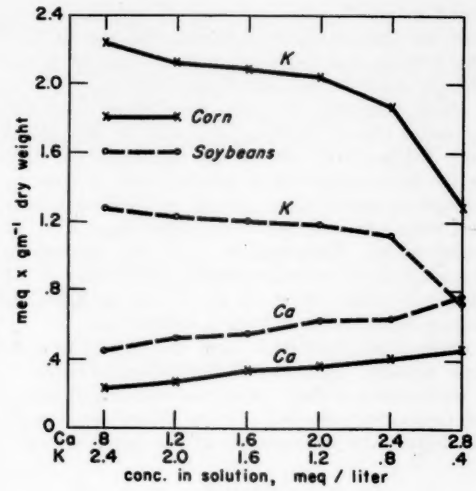
The use of Rb^{86} as a tracer for K, as suggested by Roberts et al (13), is believed to introduce no appreciable error. To verify this, experiments were con-

Table I

Rb^{86}/K Uptake by Excised Corn Roots from K Solutions Labeled with Tracer Rb^{86}

Hrs	K, meq/l	Ca, meq/l	Rb^{86}/K
	K by conductivity change *		
2nd	1	...	0.97
3rd	1	...	0.97
4th	1	...	0.97
5th	1	...	0.93
	K by flame photometer **		
0–1.5	0.8	...	1.01
0–1.5	0.8	0.5	0.91
0–1.5	1.6	...	1.02
0–1.5	1.6	0.5	1.07
0–3	0.8	...	1.00
0–3	0.8	0.5	0.98
0–3	1.6	...	0.99
0–3	1.6	0.5	0.95

* Uptake measured by loss from 1 meq/l KCl.
** Uptake measured by loss from solution with cation concentration as indicated, and equimolar Cl and PO_4 as anions (pH 5.6).



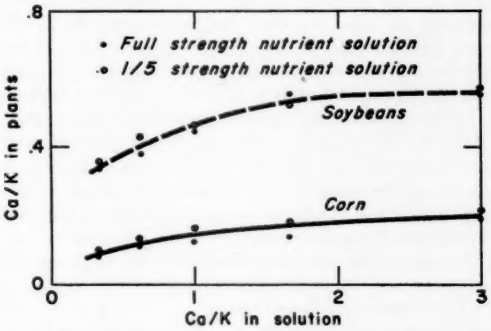


Fig. 1 (*above*). Ca and K composition of shoots of corn and soybeans grown together in nutrient solutions of varying Ca/K ratio for 4 wks.

Fig. 2 (*below*). Variation in the Ca/K ratio in the shoots of corn and soybeans as a function of Ca/K ratio in the nutrient solution.

ducted in which, after a period of equilibration to allow for completion of exchange, the loss of Rb^{68} from solution was compared with the loss of K and with the decrease in conductivity (table I).

Flask containing the roots were placed on a unidirectional shaker, shaking at a rate of 100 cycles/min. Temperature control was maintained by having the flasks immersed to solution level in constant temperature water baths. At the end of 3 hours solutions were removed and roots rinsed once with deionized water. Exchangeable Rb^{86} was removed by adding 50 ml of solutions identical with those of the absorption period except for the absence of Rb^{86}, and keeping the flasks for 1 hour in an ice bath.

Roots from each flask were placed in Coors #1 china capsules, digested with HNO_3, and radioactivity determined directly from the capsules. Conversion factors for calculation of ion absorption were obtained from the radioactivity of the initial solutions.

Results

The nutrient culture experiments show that corn and soybeans exhibit cation accumulation characteristic of their families, in that corn plants accumulate more K and less Ca than soybeans growing in the same solution (fig 1). Altering the Ca/K ratio of the solutions produced no significant difference in plant growth during a 4-week period or in the Mg content of either species and these values are not reported. If the Ca/K ratio of the solution is plotted against the Ca/K ratio in the plant, it is made apparent in the change in slope of the curves that relative cation accumulation in soybeans is somewhat more sensitive to changes in the composition of the solution (fig 2). This figure also shows that the Ca/K ratio in the plants is not much altered by a five-fold change in nutrient solution concentration.

The effect of increasing Ca concentration on K accumulation by the excised primary roots is shown in figure 3. The corn roots exhibit a typical Viets effect, in that Ca has increased K accumulation at this

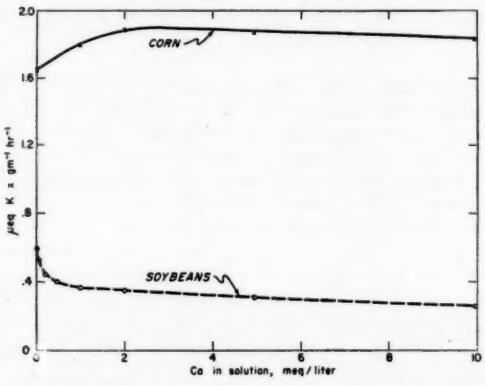


Fig. 3. The effect of increasing amounts of Ca on K accumulation by excised corn and soybean roots from 0.001 M KCl.

Table II

The Effect of Temperature Changes on K_s and V_m of K Accumulation in Corn and Soybean Roots

Plant	Ca, 0.5 meq/l	Temperatures, °C		K_s		V_m	
		Low	High	Low T	High T	Low T	High T
Corn	–	20	26	0.42	0.39	1.09	2.10
Corn	+	20	28	0.19	0.19	0.77	2.11
Corn	+	23	31	0.19	0.20	1.46	1.99
Soybean	+	25	27	0.45	0.45	1.18	1.30

K concentration (1 meq/l); however, even minute amounts of Ca depressed K accumulation by soybean roots.

In order to study the kinetics of accumulation, the experiments were carried out in K concentrations between 0.4 and 2.0 meq/l, with or without 0.5 meq/l Ca. The results were then evaluated by the method proposed by Hanes (9), plotting S/V vs S, where S = K concentration in solution in meq/l and V = K accumulation in $\mu eq \times hr^{-1} \times gm^{-1}$ fresh weight roots. V_m = 1/slope, $K_s = V_m \times$ ordinate intercept. The regression lines were calculated by least squares.

The results of these experiments (figs 4, 5) show that the K_s for K accumulation is higher in soybeans than in corn, while V_m is lower. The effect of Ca is to lower K_s and V_m in both species. Mg has a similar but smaller effect (fig 6). The small values for K_s represent a greater affinity between the K ion and the postulated carrier.

If the four regression lines of figures 4 and 5 are plotted as V vs S (fig 7), typical curves illustrating the promoting influence of Ca are obtained. Overstreet et al (12) have reported similar curves for barley roots. The promotion of K accumulation by Ca at low K concentrations follows directly from the greater affinity between the ion and the carrier. Inasmuch as K_s is lowered by Ca proportionally less in soybean than in corn roots, with proportionally greater effect on V_m, the Viets effect in soybean roots is smaller and is limited to very low K concentrations. For this reason it was not detected in earlier experiments (fig 3).

The values for K_s and V_m were determined at different temperatures (table II). While V_m had a large temperature dependence, K_s remained essentially constant.

Discussion

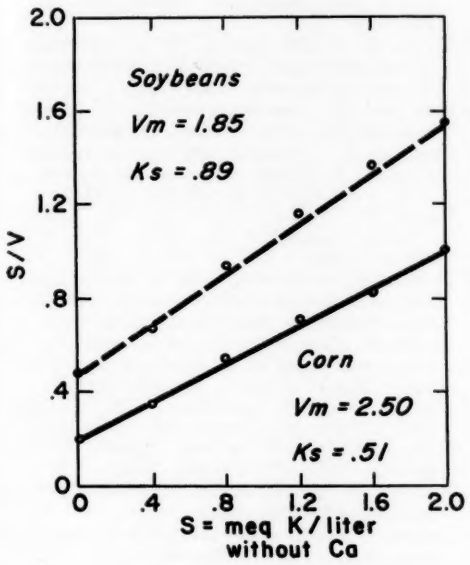
The kinetic treatment of ion accumulation introduced by Epstein and Hagan is based on the principles and assumptions of classical enzyme kinetics. The reaction for an ion M and an ion carrier R, which is metabolically changed to R′ while releasing M as an accumulated ion, is pictured thus:

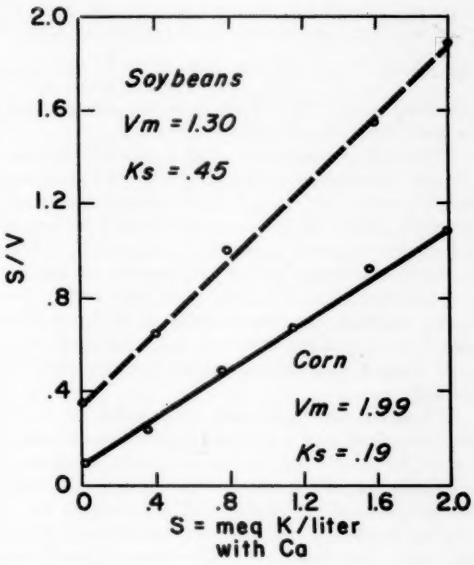
$$M_{outside} + R \underset{k_2}{\overset{k_1}{\rightleftharpoons}} MR \xrightarrow{k_3} M_{inside} + R'$$

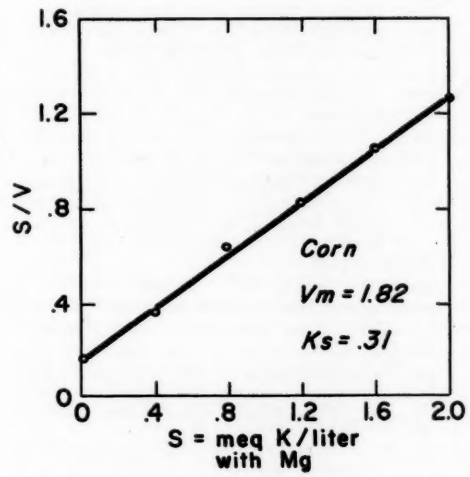
At a steady state of ion accumulation the concentration of MR will be constant, and it can be shown that the Michaelis constant,

$$K_s = \frac{k_2 + k_3}{k_1}$$

An assumption in these reactions is that k_3 is small as compared to k_2; that is, the reaction $MR \rightarrow M + R'$ is the rate-limiting reaction, depending upon metabolic energy and essentially irreversible due to large







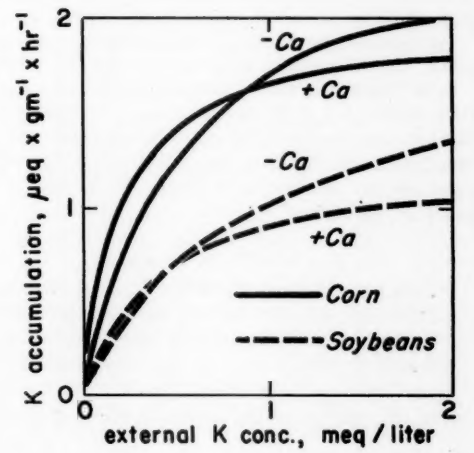


FIG. 4 (*upper, left*). Graphic kinetic analysis of K accumulation by excised corn and soybean roots in the absence of Ca. S = solution concentration; V = rate of K accumulation as $\mu eq \times gm^{-1} \times hr^{-1}$; V_m = maximum rate; K_s = Michaelis constant as meq/liter. Values at S = 0 are calculated intercepts.

FIG. 5 (*upper, right*). Same as figure 4 but in the presence of 0.5 meq Ca/liter.

FIG. 6 (*lower, left*). Same as in figure 4, for corn roots only, in the presence of 0.5 meq mg/liter.

FIG. 7 (*lower, right*). Rate of K accumulation by excised corn and soybean roots as a function of external K concentration. Curves derived from regression lines of figures 4 and 5.

negative free energy change. With this assumption, that $k_3 \ll k_2$, K_s approaches the true equilibrium constant for $M + R \underset{k_2}{\overset{k_1}{\rightleftharpoons}} MR$. Evidence for the validity of this assumption can be found in table II, where K_s proves to be constant despite large changes in V_m induced by temperature variations. If k_3, which will change with changes in metabolic rate, were relatively large, K_s should have a large temperature dependence. Since it has not, $K_s \approx \frac{k_2}{k_1}$ and is approaching a true dissociation constant, independent of the metabolic factors which influence V_m.

With this assumption, that K_s closely represents the true dissociation constant of the ion-carrier complex, the effect of Ca on K accumulation seems to be two-fold. First, Ca increases the affinity between the K ion and its carrier (lower K_s) and second, it lowers V_m by a reaction which is independent of the reaction affecting K_s. Calcium could conceivably lower V_m by lowering the concentration of R, or by lowering the rate of carrier turnover, k_3. The kinetic treatment in itself does not distinguish between these possibilities.

Whatever the mechanism, it should be recalled that the effect of Ca in promoting ion uptake is not specific for K but has been observed for bromine and phosphate as well (10, 16); in addition Ca can be substituted to some degree by Mg and other di- and polyvalent ions (7, 16). It appears that divalent or polyvalent ions must generally serve to increase the affinity between some ions and their carriers, and at the same time inhibit some phase of carrier metabolism in a manner which is not competitive.

At moderate levels of K concentration, the presence of Ca will promote K uptake in corn and inhibit it in soybeans (fig 3). This phenomenon will partially explain the relatively high K/Ca accumulation ratios in corn as compared to soybeans; however, it should be noted that even in the absence of Ca, soybeans are less efficient in K accumulation due to a higher K_s and lower V_m.

Summary

A study has been made of the kinetics of K accumulation by corn and soybean roots as affected by Ca. For both kinds of roots, Ca increases the affinity between the K ion and the postulated carrier; in a second reaction, independent of the first, Ca decreases the velocity of the metabolic phase of K uptake. The first effect of Ca is proportionally greater in corn, while the second is proportionally greater in soybeans. The net result of the two effects is to reduce maximum K accumulation in the presence of Ca more in soybeans than in corn.

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SODIUM AND SILICON AS NUTRIENTS FOR THE TOMATO PLANT[1]

JOSEPH T. WOOLLEY[2]

DEPARTMENT OF SOILS AND PLANT NUTRITION, UNIVERSITY OF CALIFORNIA, BERKELEY, CALIFORNIA

The possible essentiality of sodium, silicon, and aluminum in the nutrition of higher plants has been the subject of controversy for many years. The fact that these elements are almost ubiquitous has made the problem of ascertaining their status extremely difficult. This study was undertaken to determine at what level any one of these three elements might be needed by the tomato plant, if it were essential for the growth and development of this plant.

Most techniques for critically investigating essentiality of nutrient elements involve the extensive use of Pyrex laboratory glassware in all phases of the investigations. That this glassware is soluble to a certain extent is well known. Wichers, Finn, and Clabaugh (8) gave figures for the solubility of Pyrex glass #774 in hot distilled water. Further, it has been the experience in this laboratory that at least part of the boron requirement of plants could be obtained from Pyrex beakers in which the plants were cultured. In view of this fact and from a consideration of the composition of Pyrex (8), it would seem possible that plants might obtain significant amounts of Na, Si, and Al from such containers. Fortunately, the increasing availability of polyethylene ware, coupled with the increasing purity of the salts available from the chemical supply houses, has provided means by which the study of the possible essentiality of these three elements has been made practical. Al was added as a treatment in these experiments because this element is one which could probably be supplied by Pyrex glassware, but no Al analyses were made.

Hodgson (3) investigated the possible essentiality of Na in the nutrition of radish, barley, and celery. Purified water and salts were used as culture solutions, and measurements of Na in the salts, culture solutions, and plants were made. Small growth increases were found when 5 ppm of Na (as the chloride) were added to the nutrient solutions. With celery, a dry weight increase of 6 % was found when 5 ppm (220 μgm atoms/liter) of Na were applied. The concentration of Na in dried celery tops which had not been given sodium as a treatment was 0.00015 % (0.065 μgm atoms/gm), while the plants receiving 5 ppm of Na contained 0.011 % (4.8 μgm atoms/gm). Radish and barley gave no yield responses at this level of Na. Using unpurified solutions, Hodgson found that the yield of celery was doubled when 60 ppm (2.6 mg atoms/liter) of Na were added to the culture solutions. He concluded that Na was not essential, but that it served some beneficial function. Interpretation of these results is difficult. The larger response when impure salts were used might suggest that Na (or chlorine) was overcoming some toxicity in the nutrient solution. While Hodgson was apparently unaware of the essentiality of chlorine for the growth of higher plants, the amounts of chlorine added as $MnCl_2$ and $FeCl_2$ were probably adequate for the amount of plant growth in his experiments, so that a chlorine effect seems unlikely. One is forced to the conclusion that Na, if required for the celery plant, would be needed in concentrations of less than 0.07 microgram atoms per gram dry weight of plant.

Raleigh (5) reported that the yield of 1-month-old table beets supplied with 19 ppm of added Si in the culture solution was almost ten times the yield of the "checks." Necrosis of roots and wilting of leaves were mentioned as symptoms of Si deficiency. Wagner (7), on the other hand, using purified salts, copper-distilled water, and paraffine-coated glass containers, found that table beets gave no response to added Si. Rice, barley, cucumber, corn, tomato, and tobacco, cultured under similar conditions, gave increased growth when Si was added.

While most studies with Al have been concerned with its toxicity, Eisenmenger (2), in study such effects, obtained slight increases in growth of tobacco when what appears to have been 8 ppm of Al were applied. Analyses showed only 1 ppm (on a dry weight basis) of Al in the roots of the plants which had received no Al treatment. Iron and boron were the only micronutrients added in this experiment.

MATERIALS AND METHODS

All water used was first distilled in a tin-lined copper still and then was redistilled in a still having a Pyrex boiling flask and a polyethylene condenser. The water in the flask was boiled very slowly so that there was considerable refluxing in the proximal end of the polyethylene condenser tube. There was therefore little possibility of the mechanical carry-over of water droplets. Each culture used about eight liters of this water during the course of the experiment.

The composition of the nutrient solution, expressed in micromoles per liter, was as follows: KNO_3, 6000; $Ca(NO_3)_2$, 4000; $NH_4H_2PO_4$, 2000; $MgSO_4$, 1000; KCl, 50; H_3BO_3, 50; $MnCl_2$, 10; $ZnSO_4$, 1.5; $CuSO_4$, 0.5; H_2MoO_4, 0.1. This solution was present in the containers at the start of the experiment and the salts were replenished once during the experiment, three weeks after the transplanting date. Each week 10 ml of freshly prepared 0.0008 M $FeSO_4$ solution were added to each 2-liter culture.

All salts other than molybdic acid were prepared by two recrystallizations. Each of these recrystallizations consisted of two operations—a rapid crystallization from a hot supersaturated solution, and a slow

[1] Received January 15, 1957.

[2] Present address: Agricultural Research Service, U. S. Department of Agriculture, 276 Davenport Hall, Univ. of Illinois, Urbana, Illinois.

crystallization as the mother liquid from the first operation was gradually cooled. It was thought that impurities which tended to be brought down with the crystals would be adsorbed onto, or occluded in, the fine, rapidly formed crystals of the high temperature crystallization, which crystals were discarded. The slow crystallization at a lower temperature would allow for more perfect, and presumably purer, crystal growth. These large crystals were used in the preparation of the nutrient solutions.

The molybdic acid was prepared by the prolonged heating at 70° C of a 10 % solution of ammonium molybdate. A white precipitate formed during this heating. This precipitate was assumed to be molybdic acid and was used as such.

Marglobe tomato seeds were used. These seeds came from plants which had been cultured in polyethylene containers and had been given purified salts and polyethylene-redistilled water. Upon analysis these seeds proved to have about 0.002 microgram atoms of Na and 0.001 microgram atoms of Si per seed. This was about one fourth as much Na and less than half as much Si as were contained in the original commercially produced seeds.

The seeds were germinated on moist acid-washed cheesecloth at the surface of a one-fifth strength nutrient solution contained in a polyethylene container, with an inverted polyethylene container as a cover and black cloth over all to keep out light. Eight days after they had been set to germinate, the plants had reached the cotyledonary stage of development. At this time (June 11, 1956) three seedlings were transplanted to each culture vessel. One week later the plants were thinned to two per vessel. All plants were harvested on July 16, five weeks after the transplanting date.

The plants were cultured in 2-liter polyethylene freezer containers with holes drilled in the lids, and were supported in position by Dacron wool. This Dacron had previously been washed with 10 % HNO_3 and 10 % HF, followed by repeated rinsing with water until the pH of the rinse water remained constant.

The experiment was carried out in a specially prepared room in the greenhouse. This room was equipped so that atmospheric contaminants were presumably almost completely excluded. The air inside the room was kept at a slight positive pressure with reference to the outdoors, so that any leakage of air would be outward. All air entering the room passed through four filters in the following order:

1. A charcoal and limestone filter to trap smog and acid gases.
2. A Fiberglass filter to trap macroscopic dust, including particles of carbon from the smog filter.
3. A Mine Safety Appliance paper filter designed to stop particulate matter as small as 0.5 microns diameter.
4. An excelsior pad, continuously wetted with distilled water.

Aeration of the culture solutions was by a continuous flow of air bubbling from a polyethylene tube into each culture vessel. Each tube had a Dacron plug at the upper end to act as a filter.

The treatments, as well as the yields and the sodium and silicon contents of the dried plant materials are shown in table I. The "minus" treatment was given no Na, Si, or Al except that which was present as unavoidable contamination. The "Elkhorn sand" treatment was used as an "A–Z" mixture to detect possible micronutrients which had not been considered specifically in the design of the experiment.

At harvest, the plants were separated into shoots and roots and were placed in perforated polyethylene bags, which were in turn placed in paper bags and left in an 80° C blower-oven for several days. The dried plant material was weighed and then ground in a Wiley mill to pass a 40-mesh sieve. A polyethylene plate was substituted for the glass plate which is normally supplied as part of the Wiley mill. The samples were stored in new, but unwashed, polyethylene bags.

Sodium analyses were made with a flame photometer after the samples had been ashed at 700° C in platinum crucibles, immediately allowed to cool, and then had been taken up in 0.16 M HNO_3. Flame photometer readings were made at 586 and 594 mμ, as well as at 589.5 mμ, the wave length of the Na peak on the instrument used. An interpolation between the 586 and 594 mμ readings provided a measure of the Na-free luminescence at 589.5 mμ, this value being subtracted from the actual 589.5 mμ reading to give a measurement of the amount of Na. Known amounts of sodium added to the dried plant material were recovered quantitatively. Concentrations of sodium as low as 0.2 microgram atoms per liter could be reliably detected by this method. With the sample sizes and dilutions used, this would be 0.005 microgram atoms of Na per gram of dried plant tissue.

For Si analyses, 30 to 100 mg of the plant mate-

TABLE I

PLANT YIELD AND COMPOSITION

TREATMENT	REPLICATES	AV YIELD, GMS DRY WT			AV Na CONC, μGM ATOMS/GM		AV Si CONC, μGM ATOMS/GM	
		SHOOTS	ROOTS	TOTAL	SHOOTS	ROOTS	SHOOTS	ROOTS
Minus	8	22.5	4.9	27.5	0.089	0.18	0.15	0.10
Na *	4	25.5	5.3	30.8	29	120	0.18	0.10
Na, Al, Si **	4	25.7	5.7	31.4	30	113	0.21	0.13
Elkhorn sand †	4	24.9	5.4	30.3	0.086	1.7	0.18	8.5

* The Na treatment is 1 millimole of NaCl per liter.
** The Na, Al, Si treatment is 1 millimole of NaCl, 3 micromoles of K_2SiO_3, and 3 micromoles of $Al_2(SO_4)_3$ per liter.
† The Elkhorn sand treatment is 0.5 gm of Elkhorn sand per liter.

rial were brought to 700° C in a platinum crucible and immediately allowed to cool. The ash was then analyzed by a modification of the method of Straub and Grabowski (6). One ml of 0.15 % H_2O_2 solution was added to each sample at the start of the analysis to prevent interference by ferrous iron. Known amounts of Si added (as Na_2SiO_3) to the dried plant material were recovered quantitatively. The lowest reliably detectable concentration of Si by this method was about 0.06 microgram atoms per liter in very dilute salt solutions, the allowable salt concentration depending on the salt. The limit of reliable detection in plant material was about 0.02 microgram atoms of Si per gram of dried tissue.

Results

Recrystallization was effective in removing much of the Na from the salts. The total Na supplied to each culture by the purified salts was only 0.5 microgram atoms, as compared with the 1.6 microgram atoms which would have been supplied by the original C.P. salts.

It was not possible to ascertain whether or not the recrystallization was effective in removing Si from the salts, because the concentration of Si in the original C.P. salts was below the reliably detectable limit. The maximum amount of Si that could have been supplied by the salts was less than 0.25 microgram atoms.

The water contained 0.00085 microgram atoms of Si and 0.025 microgram atoms of Na per liter.

The total amounts of Na and Si knowingly supplied to each "minus" culture are shown in table II. The figures given in these tables for "polyethylene container" are a total of the element in question that could possibly have been supplied by the entire amount of polyethylene constituting the container. It is doubtful that more than an extremely small fraction of this was actually available to the plants.

Tables III and IV show the Na and Si supply-recovery balances. Despite the great care that had been taken to prevent contamination, it is obvious from these tables that the "minus" treatment plants received significant amounts of Na and Si from unknown sources. If the Na and Si that could possibly have been supplied by the polyethylene containers were omitted from consideration (as they probably should be), the amounts of contamination from unknown sources would become very impressive, amounting to twice the Na supply and ten times the Si supply from the remaining known sources.

The mean yield of all plants given additional Na was about 12 % greater than the mean yield of the "minus" treatment plants. This difference was significant at the one percent level. A slightly smaller difference between the "minus" yield and the "Elkhorn sand" yield was significant at the 12 % level.

The addition of one millimole of NaCl per liter of nutrient solution gave large increases in the Na concentrations in both the roots and the shoots.

Si and Al together in the culture solutions, each added at the level of three microgram atoms per liter of solution, had no effect on yield.

The Elkhorn sand treatment increased the concentration of Na in the roots, but this increase could be explained by the assumption that minute grains of sand had adhered to these roots. This explanation is substantiated by the fact that the roots receiving Elkhorn sand were the only tissues in the entire experiment which showed significantly increased concentrations of Si.

Table II

Sodium and Silicon from Known Sources

Source	Total Na, μgm atoms/culture	Total Si, μgm atoms/culture
Salts	0.511	<0.247
Water	0.20	0.007
Seeds	0.006	0.003
Dacron	0.03	0.003
Polyethylene bag	0.10	0.06
Polyethylene container	2.0*	0.25*
Total	2.847	<0.570

* This is the total amount of Na or Si in the polyethylene itself. Probably very little is actually available to the plants.

Table III

Sodium Supply-Recovery Balance

Treatment	Known Na supplied	Av Na in plants		Av Na remaining in solutions	Na recovered
		Shoots	Roots		
	μgm atoms/culture				
Minus	2.9*	2.0	0.88	0.27	3.15
Na	2000	740	640	510	1890
Na, Si, Al	2000	770	640	470	1880
Elkhorn sand	6.6*	2.1	9.2	0.29	11.6

* These values are probably excessive, since they include the entire 2 μgm atoms that could possibly have been supplied by the polyethylene container.

Table IV

Silicon Supply-Recovery Balance

Treatment	Known Si supplied	Av Si in plants		Av Si remaining in solutions	Si recovered
		Shoots	Roots		
	μgm atoms/culture				
Minus	0.57*	3.3	0.49	0.52	4.3
Na	0.57*	4.7	0.53	0.56	5.8
Na, Si, Al	6.6	5.4	0.75	1.68	7.8
Elkhorn sand	**	4.6	46	9.65	60.3

* These values are probably excessive, since they include the entire 0.25 μgm atoms that could possibly have been supplied by the polyethylene container.

** The Elkhorn sand was not analyzed for Si.

Discussion

The yield increase with the Na treatments could have been a direct response to the added Na. There are, however, several other possible explanations.

Firstly, the increase in yield may have been a response to the one milligram atom of chlorine which was added with the sodium (NaCl). Experience in this laboratory indicates that plants show slight growth increases in response to comparatively large chlorine additions even when the original chlorine level is as high as 50 microgram atoms per liter, as it was in this experiment (1). This explanation would seem doubtful, however, in view of the fact that the amount of Elkhorn sand added could have supplied only 2 microgram atoms of chlorine per liter, yet this treatment also was accompanied by an increase in growth.

Secondly, the potassium supply could have been inadequate, so that the Na was substituting for some of the required K. This explanation, too, would be doubtful in view of the increase in growth with the Elkhorn sand. The 20 microgram atoms of Na and K combined contained in the Elkhorn sand would be insignificant in comparison with the 24 milligram atoms of K which were present in all treatments. In fact, the entire 2 milligram atoms of Na which were added as the Na treatment, if used in substitution for K, could account for an increase of only 8.3 % in the total Na and K combined. This would be insufficient to give the 13 % increase in yield observed with the Na treatments, especially considering the fact that about one fourth of the Na supplied in these treatments remained in the culture solution (table III).

Thirdly, there remains the possibility that the yield increase is a response to some impurity in the NaCl. Further experiments with different Na salts and different purification methods would be necessary to show whether this is the correct explanation.

In general, this work confirms the results of Hodgson. The level of Na found in the "minus" plants and the yield responses to added sodium are comparable to those found by Hodgson with celery plants. Hodgson also found inadvertent contamination levels similar to those encountered here.

The most likely (though by no means the only possible) explanation for this increase in yield would seem to be that the increase was a direct effect of the Na, which was performing some specific function within the plant. Before any final conclusions as to the essentiality of sodium could be drawn, however, larger effects would have to be observed in a series of experiments designed to eliminate all other possibilities.

From the present work, the only tenable conclusion is that Na, if essential to the tomato plant, would be required only in concentrations of less than 0.10 microgram atoms per gram dry weight of plant. This would correspond with about 2.5 microgram atoms of Na per liter of the usual culture solution. If Na were essential at such a level, the Na requirement would be roughly comparable with that of manganese.

Added Si did not increase the growth of the tomato plants. The experiments showed nothing to indacte that the added Si actually made its way into the plants, as there was no significant increase in silicon concentration in the shoots regardless of the treatment. It is possible that all of the Si found either was in the form of dust on the leaves or was associated with the root surface. Consequently, it may be that the actual Si levels within the plant were much lower than were found in this work.

It is concluded that Si, if essential to the tomato plant, would be required only in concentrations of less than 0.2 microgram atoms per gram dry weight of plant.

No Al analyses were made, because it seemed doubtful that available methods of Al analysis would have the required sensitivity when used on plant material. It seems reasonable to suppose, however, that the amount of Al supplied to the plants from all sources was no greater than the amount of silicon so supplied. If such a supposition is valid, it would seem that Al, if essential, would be needed by the tomato plant in concentrations of less than 0.2 microgram atoms per gram dry weight of plant.

Considering the good growth that obtained when extreme care was taken to exclude Na, Si, and Al from the environment of the "minus" plants, and considering the virtual ubiquity of these elements, it would seem doubtful that the true deficiencies of any one of these three elements would ever be encountered in agricultural practice. It is probable that the beneficial yield effects often observed from the application of Na to soils are caused by some indirect action of this element, or possibly by the accompanying anion.

Further investigation of the possibility of essentiality of Na, Si, and Al would be appropriate, but the large amounts of inadvertent contamination met with in these experiments and in Hodgson's similar work would indicate that further purification of salts and water is unlikely to be fruitful in ascertaining essentiality of these elements until other contamination sources have been identified and eliminated.

A more detailed report of this work can be found elsewhere (9).

Summary

Tomato plants were grown in solution culture in polyethylene vessels in a greenhouse which was equipped with air filters. Nutrient salts were purified by recrystallization. Water used was redistilled in polyethylene.

The plants showed a 12 % increase in dry weight with additions of sodium at the rate of one millimole of NaCl per liter of culture solution. No increase in yield was shown with addition of 3 microgram atoms of silicon and 3 microgram atoms of aluminum per liter of culture solution.

Sodium was measured by the flame photometer and was present at a level of 0.09 microgram atoms

per gram of dried plant shoot material when no sodium was intentionally added.

Sicilon was measured as silicomolybdate. The silicon concentration in the dried plant shoot material was 0.15 microgram atoms per gram when no silicon was intentionally added.

Aluminum was not measured.

It was concluded that if sodium or silicon were essential for the growth and development of the tomato plant, sodium would be required in amounts of less than 0.1 microgram atoms, and silicon would be required in amounts of less than 0.2 microgram atoms, per gram dry weight of plant.

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A COMPARISON OF THE LIGHT DEPENDENT METABOLISM OF CARBON MONOXIDE BY BARLEY LEAVES WITH THAT OF FORMALDEHYDE, FORMATE AND CARBON DIOXIDE[1,2]

A. R. KRALL[3] AND N. E. TOLBERT

Biology Division, Oak Ridge National Laboratory, Oak Ridge, Tennessee

Fixation by plants of CO_2 into photosynthetic products in the light or exchange in carboxylation reactions in the dark has been the subject of many investigations with C^{14}. Formic acid metabolism also has been studied in plants (11, 14), but the fixation and metabolism of two other C_1 compounds, carbon monoxide and formaldehyde, have not been extensively studied.

Few effects of CO on living systems, other than inhibition of oxidative enzymes, have been recorded. Bottomley and Jackson (3) reported that nasturtium leaves produced starch from CO in a CO_2-free atmosphere when illuminated. Crocker (5) found that tomato plants exposed to low concentrations of CO, 0.1 to 1.0 %, produced a profuse growth of adventitious roots within a week. Fenn and Cobb (7) found that CO was converted to CO_2 by isolated tissues. Clark (4) found that this was accomplished by intact mice and turtles when the CO was present at low concentrations and that C^{14} from radioactive CO appeared in CO_2 at the same rate at which gasometric analysis indicated the CO was disappearing from the atmosphere. Daly (6) observed a stimulation of the respiration of wild plum leaves by CO which was shown by isotopic methods not to be the result of oxidation of CO to CO_2.

This paper reports uptake of C^{14} from radioactive CO by cut barley leaves at about the same rate, on a fresh weight basis, at which Clark (4) reported that mice consumed CO. The process is light dependent and the major soluble C^{14}-labeled product is serine.

Numerous suggestions that formaldehyde was a first product of photosynthesis are to be found in older reviews on photosynthesis, but this concept has not been documented by data and in fact relatively small amounts of formaldehyde are toxic to plants. Nevertheless formaldehyde-C^{14} feeding to animals has shown that it is a C_1 donor (13). It has recently been shown that $H_2C^{14}O$ is fixed at a very slow rate in darkness by barley leaves into such products as serine and choline which are not products of the photosynthetic cycle. At high light intensities (2000 ft-c) the fixation proceeds at several fold the dark rate and a large share of the label goes into sucrose and glyceric acid as well as into serine and choline (11).

Material and Methods

C^{14}-labeled CO was prepared from $BaC^{14}O_3$ by a modification of the zinc reduction method (2) or by dehydration of C^{14}-formic acid (furnished by Dr. G.

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[2] This work was performed under U. S. Atomic Energy Commission contract No. W-7405-eng-26.

[3] Present address: RIAS Inc., P. O. Box 1574, Baltimore 3, Maryland.

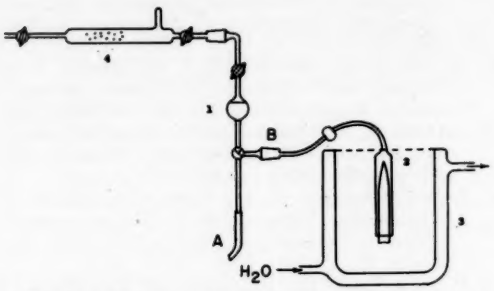


FIG. 1. Glassware for the conversion of $C^{14}O_2$ to $C^{14}O$ and for fixation of $C^{14}O$ by single barley leaves. 1. Scrubbing chamber containing 3 to 4 *N* KOH. 2. Flattened gas exposure chamber 8 mm wide × 2.5 mm thick × 11 cm long. 3. Water cooling bath. 4. Reaction vessel.

A. Ropp) with concentrated sulfuric acid. In the first method $C^{14}O_2$ was slowly passed through an ethanol–dry ice trap to remove water vapor and then collected in a liquid nitrogen cooled side arm of the evacuated reaction vessel (4, fig 1). About 1 ml (at NTP) of $C^{14}O_2$ containing 25 % C^{14} was used. The reaction vessel was heated for 3 hours at 400° C. The $C^{14}O$ which was formed was then displaced by mercury into a scrubbing chamber (1, fig 1), containing 4 *N* KOH, to remove all unreacted $C^{14}O_2$. Less than one percent of the activity was retained by the alkali. If, however, the CO were allowed to stand over the alkali for several days, the C^{14} was completely absorbed. This slow uptake by alkali is characteristic of CO (9).

First leaves of barley, weighing from 95 to 105 mg, were used in all the experiments. They were cut and transferred immediately to the exposure chamber, which was filled with water at this time. The $C^{14}O$ gas was then tranferred from the scrubbing chamber to the exposure chamber by rotating 1 about joint B (fig 1) so that it was below B. The gas could be transferred from chambers 1 to 2 at will and could be readily freed of any $C^{14}O_2$ produced by the plant. The extremely low rate of $C^{14}O$ uptake by the plant permitted several experiments with the same gas without introducing an error from changes in concentration. The gas used in the experiments contained 60 % $C^{14}O$, 8 % oxygen and 32 % nitrogen.

The leaves were illuminated at different intensities by fluorescent lights or incandescent flood lamps. The water bath in which the exposure chamber was immersed was kept at a constant temperature (about 12° C) within any one exposure. At the end of an exposure the leaf was removed from the bottom of the exposure tube with tweezers and extracted in boiling 30 % ethanol.

Formic acid (11) and formaldehyde fixation experiments (unpublished, Tolbert and Krall) were run by feeding the cut leaf the labeled compound in solution.

An aliquot of the extracts was dried and counted for total C^{14} fixed and another aliquot chromatographed on Whatman No. 1 paper in two directions using water-saturated phenol as the first developer and a butanol–propionic acid–water (1) as the second. C^{14}-labeled products were located by radioautography and the distribution of radioactivity among these products determined by direct counting of the radioactive spot with a thin window G-M tube.

Compounds on the chromatograms were identified by their R_f values and by cochromatography with carrier. The identification of phosphoryl choline was described by Maizel et al (8).

RESULTS

In the dark only minute traces of $C^{14}O$ were fixed (table I). Figure 2 A shows the C^{14} distribution from $C^{14}O$ uptake in the light. Serine contained about half the isotope fixed from $C^{14}O$ while considerable label was present in phosphate esters and small amounts in sucrose, glycine and glycolic acid. The high percentage incorporation of C^{14} into serine from $C^{14}O$ resembles the distribution found with $HC^{14}OOH$ (fig 2 B) or $H_2C^{14}O$ (fig 2 C). The latter two compounds in addition labeled phosphoryl choline, choline and aspartic acid. In the light $HC^{14}OOH$ yielded a large amount of label in sucrose and glyceric acid (11) unlike the C^{14} distribution from $C^{14}O$ in the light. $H_2C^{14}O$ metabolism in the light was similar to its metabolism of HCOOH in light, and the fixation was several fold faster in the light. This increased fixation rate in the light was also observed with formic acid (11). Dark fixation of $H_2C^{14}O$ was slow but gave products very similar to those found with $C^{14}O$ in light (fig 2 D).

Fixation of $C^{14}O_2$ in the dark was into products of the citric acid cycle. In the light, $C^{14}O_2$ was fixed into the compounds of the photosynthetic carbon cycle, figure 1 A (11). If the partial pressure of $C^{14}O_2$ was made very low so that C^{14} was fixed in the light at about the same rate from $C^{14}O_2$ as it was fixed from $C^{14}O$ or $H_2C^{14}O$, only sucrose accumu-

TABLE I

RATE OF UPTAKE OF LABELED $C^{14}O$ BY BARLEY LEAVES

No.	LIGHT INTENSITY, FT-C	TIME, MIN	RATE *
1	10	990	490
2	100	300	650
3	5,000	80	670
4	0	120	17
5	20	27	405
6	600	25	470
7	2,000	45	363
8	2,000	80	470
9	10,000	39	390

* Rate is expressed as counts per sec C^{14} fixed per leaf per hr exposure to labeled $C^{14}O$. Leaves varied in weight from 95 to 105 mg. The first three experiments were done with one set of barley leaves, the rest with another set.

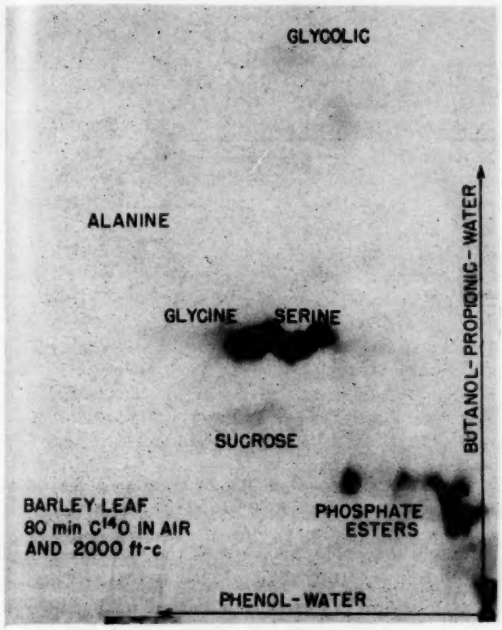


FIG. 2 A. Chromatographic separation of the products of fixation of $C^{14}O$, 80 min, 2,000 ft-c, 12° C.

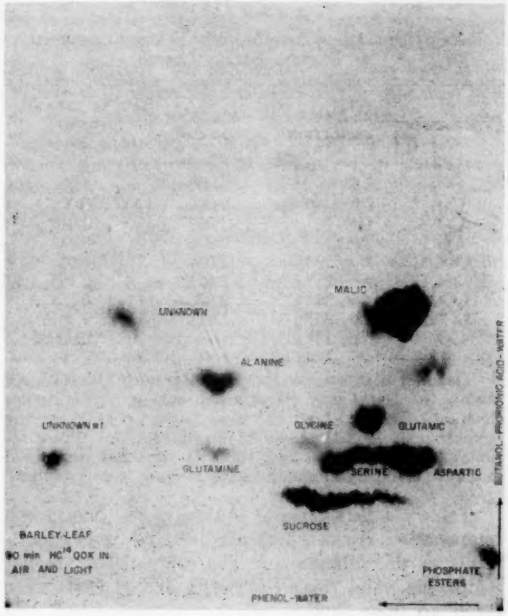


FIG. 2 B. Chromatographic separation of the products of fixation of $HC^{14}OOH$, 90 min, 2,000 ft-c, 23° C.

lated appreciable amounts of C^{14} from the $C^{14}O_2$. The presence of CO does not affect the pathway of $C^{14}O_2$ uptake even though in some cases (10) it has been shown to retard the rate of uptake momentarily.

Table I shows the amount of $C^{14}O$ fixed by the leaves at different light intensities and for different lengths of time. In darkness only a very small amount of $C^{14}O$ was fixed. The uptake of $C^{14}O$ appeared to proceed at a nearly constant pace for long periods of time in the light and was not appreciably affected by a rise in light intensity above 10 ft-c.

Table II shows the percentage distribution of C^{14} among the various labeled products in $C^{14}O$ metabolism. Serine contained the most label in all cases but the last. If labeled carbon dioxide were assimilated for this length of time about half the label would be in sucrose. Percentages are not listed for the dark experiment because the rate of uptake was so low that it was impossible to get an accurate count within a reasonable time on the faint spots present. The differences between high and low light intensities are that at the higher light intensity the phosphate area contained less C^{14} and showed a wider distribution of C^{14} as evidenced by a larger number of phosphate ester spots, and by the appearance of more label in spots summed in the other column. Line 5 in table II shows the effect of infiltrating 0.0001 M 2,4-dinitriphenol (DNP) into the leaves before exposure to $C^{14}O$. The pattern of C^{14} distribution was almost exactly like that given by fixation of $C^{14}O_2$ in the light. The rate of uptake of $C^{14}O$ was not significantly lowered by the DNP.

TABLE II

DISTRIBUTION OF C^{14} IN PRODUCTS OF $C^{14}O$ UPTAKE BY BARLEY LEAVES

EXPTL CONDITIONS	EXTRACTABLE PRODUCTS, %						
	SERINE	GLYCINE	SUCROSE	GLYCERIC ACID	ALANINE	PHOSPHATE AREA	OTHER
Dark, 12° C, 120 min	Trace	..	Trace	..	..	Trace	..
20 ft-c, 12° C, 27 min	42	3	3	3	10	37	3
600 ft-c, 12° C, 25 min	47	8	17	Trace	Trace	22	5
2,000 ft-c, 12° C, 80 min	41	3	7	3	3	29	15
2,000 ft-c, 12° C, 45 min *	10	21	38	Trace	Trace	29	2

* This leaf was infiltrated with 1.5×10^{-4} M DNP before the experiment and then placed in the dark in an air stream until it regained normal appearance.

TABLE III

RATE OF UPTAKE OF DIFFERENT 1-CARBON COMPOUNDS BY EXCISED BARLEY LEAVES

COMPOUNDS	EXPTL CONDITIONS	LIGHT, FT-C	RATE OF UPTAKE, MICROMOLES/GM × HR
CO_2	0.05 % in air	2,000	13.0
CO_2	2.0 % in air	2,000	760.0
CO	12° C	2,000	0.018
CO	28° C	2,000	0.038
HCOOH	Air, 29° C	2,000	1.05
HCOOH	Air, 23° C	Dark	0.105
H_2CO	Air, 23° C	2,000	0.168
H_2CO	Air, 23° C	Dark	0.0011
CO	Air, 12° C	Dark	0.00065

The first 4 experiments were done with Oderbrucker barley, the last 4 with Sacramento barley. Weights are fresh wt. The CO_2 and CO were taken up as gases, the formate and formaldehyde from solution by transpirational pull. About 50 μgm substrate/gm leaf were used in the formaldehyde and formate feeding experiments.

Table III lists rates of uptake of the one-carbon compounds for which data are available from experiments in this laboratory. The comparisons are not completely valid since $HC^{14}OOH$ and $H_2C^{14}O$ were taken up from aqueous solution whereas $C^{14}O_2$ and $C^{14}O$ were absorbed in the gaseous form. The figure for formic acid uptake in light is minimal since most of the formate was metabolized, while a large excess of the other substrates was present at the end of those experiments. The order of reactivity in fixation by barley leaves under the conditions listed were:

$$CO_2 \gg HCOOH \geqslant H_2CO > CO$$

DISCUSSION

Only a very low level of light (10 to 20 ft-c) is required to attain maximum rate of $C^{14}O$ uptake, though fixation in the dark is negligible. The C^{14} from CO is fixed mainly into serine. In contrast $C^{14}O_2$ fixation is maximal at light intensities 50 to 100 fold higher and it mainly labels sucrose and phosphate esters of the photosynthetic carbon cycle. The maximum observed rate of $C^{14}O$ uptake was only 0.002 % that of CO_2 uptake, which rate is so low as to be unobservable by Daly's method (6).

Fixation of C^{14}-labeled CO, formaldehyde and formate resemble one another in that serine is a major product from each. The products are different in that $H_2C^{14}O$ and $HC^{14}OOH$ caused the plant to produce fairly large amounts of C^{14}-labeled phosphoryl choline, choline, and aspartic acid. The reason for these differences is not known. It may be that CO is converted to serine by a process not involving the same C_1 precursor used for formation of methyl groups of choline. Excess CO may also have inhibited the labeling of choline. Alternatively, the higher concentration of the C_1 precursors furnished by formate and formaldehyde by the aqueous solutions in which they were fed may have promoted labeling of choline, which could not occur at the presumably low concentration of precursor formed from CO gas.

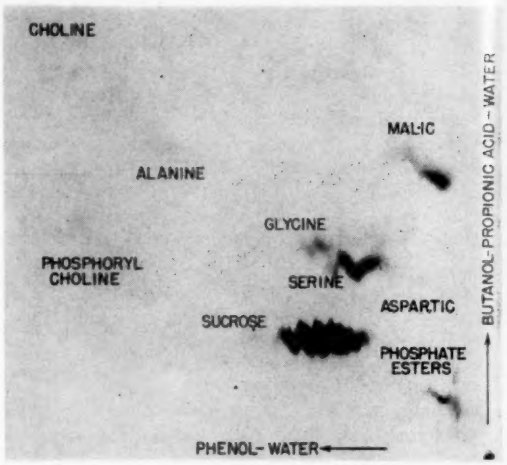


FIG. 2 C. Chromatographic separation of the products of fixation of $HC^{14}O$, 60 min, 2,000 ft-c, 23° C.

DNP inhibition causes a shift in the products usually obtained from the metabolism of formaldehyde or formate to those obtained from photosynthetic fixation of CO_2. Since the conditions here (no CO_2) are favorable to the maintenance of a high level of ribulosediphosphate, which level, in turn, is dependent on adenosinetriphosphate (ATP) level, and since ribulosediphosphate can be converted to glycine in vitro (12), it would be logical to conclude that a drop in level of ATP caused by DNP infiltration could result in a drop in glycine level and consequently in a lowered rate of serine synthesis.

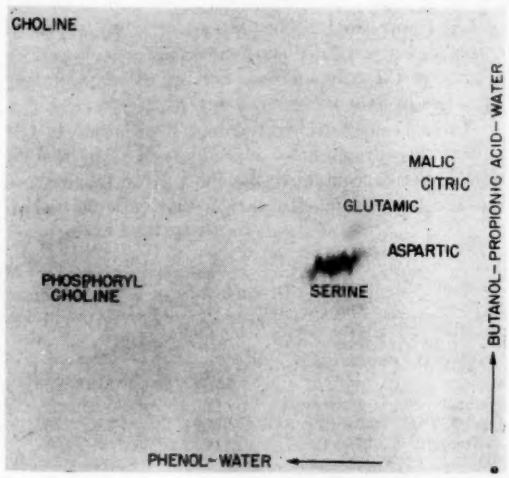
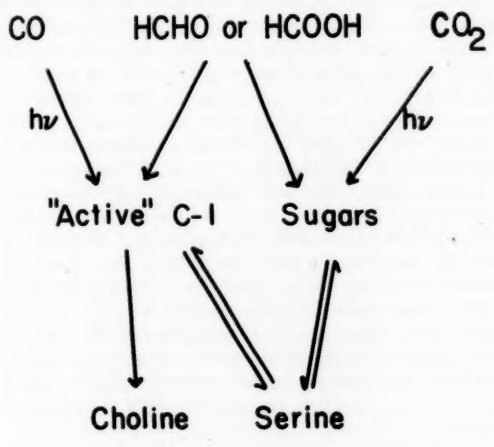


FIG. 2 D. Chromatographic separation of the products of fixation of $H_2C^{14}O$, dark, 23° C.

The level of illumination required for $C^{14}O$ fixation is very similar to that necessary for photodissociation of an iron-carbonyl complex. It may be that in this process some of the CO is reduced in a manner analogous to that in which cytochrome oxidase reduces oxygen. This reduced CO might then be the "active" molecule which appears in serine. This hypothesis can be tested by determining the action spectrum of $C^{14}O$ uptake.

The rate of labeling of the plant by $C^{14}O$ in the light or in dark was somewhat slower than by $H_2C^{14}O$ under the same conditions. At higher light intensities $H_2C^{14}O$ incorporation was stimulated to about the same extent as was fixation of formic acid. Both formic acid and formaldehyde were fixed in the light very slowly compared with rate of uptake of CO_2.

On the basis of the data presented here the following interrelationships appear to exist between the C_1 compounds studied: Formaldehyde and formate are roughly equivalent to one another, the slight differences in rate of uptake in bright light probably being the result of either concentration differences or inhibitory effects of the formaldehyde. The distribution of label from uptake of CO most closely approximates that from these two compounds except that it is not incorporated into choline. It may be that CO is never converted to the "active" C_1 group used for choline synthesis or that this compound is never present in high enough concentration to promote choline synthesis. No comparison between the affinity constants for C_1 precursors of the serine and choline synthesizing enzymes from other systems are known. Carbon dioxide metabolism is not related to that of the other three one-carbon units except at high light levels. It never labels phosphoryl choline and therefore does not seem to be related to or reduced to the level of formaldehyde or formate. These compounds do, however, under certain conditions, seem to be oxidized to carbon dioxide and photosynthetically incorporated as such. The following diagram best expresses these relationships:



Summary

Comparison has been made of uptake of four different 1-carbon compounds by barley leaves as to rate and products of fixation using C^{14}-labeled substrates. Carbon monoxide uptake, although very slow, is found to be light dependent. It is incorporated mainly into serine and secondarily into products of the photosynthetic carbon cycle. 2,4-Dinitrophenol inhibition shifted the carbon monoxide pattern to one resembling carbon dioxide uptake. It is suggested that reduction of carbon monoxide takes place on cytochrome oxidase at the time of photodissociation of its complex with the enzyme.

Formaldehyde-C^{14} fixation in the dark is slow but in the light it is metabolized several fold more rapidly. Formaldehyde and formate are fixed into serine, choline and phosphoryl choline in both light and dark. In light formate and formaldehyde are also fixed rapidly into products similar to those found from carbon dioxide while carbon monoxide is converted to these much more slowly. Formate uptake is more rapid than that of either formaldehyde or carbon monoxide.

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STUDIES ON THE FORMATION OF ASCORBIC ACID IN DETACHED APPLE LEAVES[1]

E. A. M. ASSELBERGS[2]
Canada Department of Agriculture, Ottawa, Ontario, Canada

Giroud et al (4) found a close relationship between light intensity, chlorophyll content and ascorbic acid content of green plant tissues, supporting the concept that ascorbic acid formation is directly related to photosynthesis. In chlorophyll-free tissues and in seedlings germinating in the dark, Reid (13) postulated that ascorbic acid was formed by in-dark conversion of a light induced precursor. This hypothesis was supported by Aberg (1) who found that more ascorbic acid accumulated in pea leaves in the dark than in the light, but that the quantity accumulated was related to the light intensity of the previous day. Similarly, Popovskaja (11), and Prokoshew (12) reported the formation in light of a precursor of ascorbic acid in the leaves of potato and tobacco plants which, transported to the tubers and fruits, is converted into ascorbic acid independently of light.

The actual precursor of ascorbic acid has been the subject of much investigation and many substances have been proposed as the precursor. Horowitz and King (6) and Mapson et al (8, 9) have recently presented conclusive evidence of two pathways of ascorbic acid synthesis in which D-glucuronic acid, D-galacturonic acid and their respective gamma lactones would be the active intermediates.

The ascorbic acid content of apples has been determined frequently, but the synthesis of ascorbic acid in apple leaves has received little attention. Because ascorbic acid synthesis in apples may be analogous to the mechanism in potato tubers and tobacco fruits, the present study was undertaken to obtain information on the precursor and the influence of light and darkness on ascorbic acid synthesis in detached apple leaves.

Materials and Methods

The apple leaves used (*Malus sylvestris* Mill., var. McIntosh and Rhode Island Greening) all came from one-year-old shoots on three-year-old root systems. To further increase the probability of obtaining uniform material, these shoots were grown in the greenhouse in quartz sand with nutrient solution applied regularly. When the terminal bud had formed, the shoot was four to five feet in height and bore 30 to 40 leaves. Only the top 20 full-grown leaves were used, each of which weighed between 0.6 and 1.6 gm and had a surface area of 20 to 70 cm^2.

The experimental techniques used by previous workers consisted of either floating whole leaves (1) or discs of tissue (2, 15) or placing germinating seedlings (8) on the desired solution. To detect changes in the ascorbic acid content of the material, resulting from experimental treatments, they determined the ascorbic acid content of representative aliquots of the treated material and water controls at zero time and at certain time intervals thereafter.

In the present study the experimental solutions were fed to detached apple leaves through the petiole. The easily manipulated woody petiole was inserted in a piece of 3 mm glass tubing, slightly tapered at the end to ensure a tight fit. Adhesive tape sealed the petiole in the tubing and completely prevented leakage of the solution at this junction. The other end of the tapered glass tube was inserted into a one inch piece of rubber tubing. With a glass tube, drawn out in a long fine point, some of the desired solution was put in the tapered glass tubing, making sure that no air bubbles were present. Subsequently a 100-ml Midvale bottle was filled with the desired solution until some of the liquid started to pour out of the side arm tube. As soon as this occurred the container unit was tilted over slightly in order to prevent further loss of solution. Holding the container unit in one hand and the leaf, attached to the tapered glass tube and rubber tubing (previously filled with solution) in the other hand, the two parts were put together while making certain that no air bubbles entered the system. This method kept detached apple leaves in a healthy condition for more than three weeks when the solution level in the container was maintained. Preliminary results (3), indicated that backward movement of solutes from the leaf into the Midvale bottle occurred only from autolyzed tissue in direct contact with the water in the container.

In preliminary experiments on sampling technique

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Contribution from the Pomology Department, Cornell University, Ithaca, N. Y. Based on part of a thesis submitted in partial fulfillment of the requirements for the degree of Doctor of Philosophy.
[2] Supported in part by a Fellowship from the Shell Oil Company.

TABLE I

VARIATIONS IN FRESH WEIGHT AND ASCORBIC ACID CONTENT IN 1-CM² DISCS OF APPLE LEAVES FROM A SINGLE TREE

LEAF NO.	DISC NO.	WT OF LEAF DISC	ASCORBIC ACID
		mg	*μgm/disc*
A	1	14.7	87
	2	14.7	86
	3	14.5	87
	4	14.6	86
B	1	13.4	62
	2	13.6	65
	3	13.5	65
C	1	17.1	67
	2	17.0	62
	3	17.2	62
	4	17.0	64
D	1	17.0	80
	2	17.0	92
	3	17.1	88
	4	17.0	94
E	1	16.8	54
	2	17.0	56
	3	17.0	57
F	1	15.1	61
	2	14.9	64
	3	15.1	61
	4	14.9	61

it was found that the ascorbic acid content of whole leaves of one tree and also of comparable leaves of different trees varied considerably. None of these variations could be explained on the basis of age, position on the tree, leaf weight, phyllotaxy or internode length. However, it is shown in table I that variations in ascorbic acid content and fresh weight of 1 cm² discs taken from one leaf are small (about 5 %) as long as care is taken to avoid inclusion of midrib or primary veins. Therefore one to three one-square-centimeter discs were cut from each experimental leaf at the beginning of an experimental run, and additional discs were cut at specified time intervals thereafter. The discs of each sample were taken from both sides of the leaf. The ascorbic acid analyses were performed immediately after each sampling and either the combined discs from each leaf or the combined discs from the leaves receiving the same treatment were analyzed each time as one sample. The results are expressed as micrograms of ascorbic acid per disc because this does not require separate weighing of the discs.

Ascorbic acid, dehydroascorbic acid and 2,3-diketo-1-gulonic acid were determined according to the method of Roe et al (14), using the "Kromatrol" colorimeter with a 525 mμ filter. Because the concentrations of the last two compounds were found to be negligible in apple leaves (3), only the combined values of ascorbic acid, dehydroascorbic acid, and 2,3-diketo-1-gulonic acid are reported.

The equipment used for studies with C^{14} labelled CO_2 consisted of an air tight glass cabinet. The four longitudinal walls (42″ high by 22″) were glass windows, one of which could be conveniently removed to place the required material in the cabinet. The top and bottom of the cabinet were made of ¾-inch plywood. Several openings were made in the top for necessary connection for a thermometer, hygrometer, pressure gauge and $C^{14}O_2$ supply. The $C^{14}O_2$ was produced by mixing a small quantity labelled $BaCO_3$ with excess lactic acid. The calculated CO_2 concentration in the cabinet at the beginning of an experimental run was 0.05 %. After exposure to $C^{14}O_2$ for certain lengths of time leaf discs were analyzed for their ascorbic acid content according to the method of Roe et al (14). Aliquots of the HPO_3 extract were used for preparing the hydrazines which were filtered off on an extra-fine glass sintered plate. The hydrazines were dried on this plate and then put in the Geiger counter (standard windowless flow counter), to determine the counts per minute. The results are expressed as counts per minute per 100 micrograms ascorbic acid and may be denoted as the "specific activity".

EXPERIMENTAL RESULTS

ABSORPTION EXPERIMENTS WITH SUGAR AND SUGAR DERIVATIVES: A number of substances were absorbed by detached apple leaves using 0.1 and 0.5 % concentrations in the Midvale bottle at low (600 ft-c) and high (1700 ft-c) light intensity for 24 and 48 hours. These substances were D-glucose, D-fructose, L-sorbose, sucrose, fructose-1,6-diphosphate, D-sorbitol, D-glucuronic acid and its potassium, calcium and sodium salt, L-gulonolactone, 2-keto-1-gulonic acid and its sodium salt and D-galacturonic acid. Glucosecycloacetoacetic acid and its ethyl ester were also included because, according to Nath et al (10) these serve as precursors of ascorbic acid in *Phaseolus Mungo*.

At high light intensity, sorbose, sucrose, fructose-1,6-diphosphate, D-galacturonic acid, glucosecyclo-

TABLE II

CHANGES IN THE ASCORBIC ACID CONCENTRATION OF DETACHED APPLE LEAVES WHEN VARIOUS SOLUTIONS WERE ABSORBED THROUGH THE PETIOLE AT 1700 FT-C AT ROOM TEMPERATURE

TREATMENT	NO. LEAVES TREATED *	AV INCREASE IN ASCORBIC ACID	
		24 HRS	48 HRS
		μgm/disc.	*μgm/disc*
D-Glucuronolactone, 0.1 %	24	14 **	16
L-Gulonolactone, 0.1 %	18	3	8
D-Galacturonic acid, 0.1 %	12	4	3
Glucose, 0.1 %	12	8	20
Fructose, 0.1 %	18	8	14
H_2O control	36	6	15

* The combined discs from each leaf at each sampling period were analyzed as one sample.

** Significantly different from H_2O control at 1 % level.

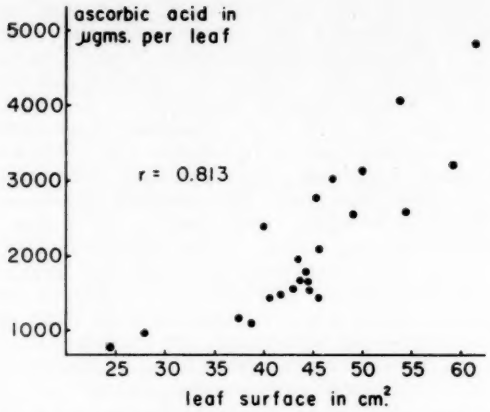

FIG. 1. Relationship between ascorbic acid content of apple leaves and their surface area. All leaves were taken from one shoot.

acetoacetic acid and its ethyl ester, 1-gulonic and 2-keto-1-gulonic acids and their sodium salts, and the calcium, potassium and sodium salts of D-glucuronic acid did not increase the ascorbic acid content of apple leaves. Moreover, the 0.1 and 0.5 % solutions of the sodium, calcium and potassium salts of the sugar acids were toxic.

Table II summarizes the experiments with 0.1 % solutions of glucuronolactone, gulonolactone, galacturonic acid, glucose and fructose. Glucuronolactone caused a significant increase in the ascorbic acid concentration after 24 hours, but gulonolactone and D-galacturonic acid, which were proposed as intermediates by Isherwood et al (7) induced less ascorbic acid snythesis than the water control although the decrease was not statistically significant. Similarly the increases in ascorbic acid concentration from glucose and fructose were not significantly greater than the water control.

Mapson et al (9) also found that L-gulonolactone caused no increase in ascorbic acid when they used cytoplasmic particles from pea seeds and cress seedlings. They suggested that the natural path of ascorbic acid synthesis is via the galacturono-galactonolactone rather than by that of glucurono-gulonolactone. However, the results of the experiments with detached apple leaves do not support the galacturono-galactonolactone pathway. Moreover, the negative results obtained with gulonolactone suggest that the active intermediate between glucuronic acid and ascorbic acid may be derivative of the sugar acid other than the gamma lactone.

RELATIONSHIP BETWEEN ASCORBIC ACID CONTENT AND SURFACE AREA OF APPLE LEAVES: In an attempt to indicate a possible relation between the ascorbic acid content of individual whole apple leaves of one tree and also of comparable leaves of different trees with respect to position on the tree, fresh leaf weight, age, phyllotaxy and internode length, a large number of ascorbic acid analyses were performed. The results of these analyses showed that the variations found in the ascorbic acid content of whole apple leaves could not be explained on the basis of age, position on the tree, leaf weight, phyllotaxy or internode length. However, representative date (fig 1) show a high correlation (r = 0.813) between ascorbic acid content of apple leaves and their surface area. This possibly reflects the influence of photosynthesis on ascorbic acid formation and confirms earlier work of Reid (13) with young cowpea plants.

RESULTS OF EXPERIMENTS WITH DIFFERENT LIGHT INTENSITIES: In initial experiments (3) changes in the ascorbic acid and dehydroascorbic acid content of detached apple leaves under low light intensity (600 ft-c) were negligible for periods up to 144 hours. Under exposure to 1700 ft-c a small increase was noticed after 48 hours. The results at low light intensity immediately prompted the suggestion that utilization equalled synthesis.

To determine this, apple leaves were subjected to C^{14} labelled CO_2 at low and high light intensities for a maximum period of six hours. The results are recorded in table III. Although only negligible changes in the ascorbic acid concentration could be detected at both light intensities, and although considerably more labelling occurred at 1700 ft-c the incorporation of C^{14} at 600 ft-c indicates that ascorbic acid was synthesized at the low light intensity. Variations between the different samples are large but are understandable in view of the work of Heinicke (5) who found in leaves from one apple tree, which were well paired as to position on the tree, weight and surface area, that only 12 of the 49 pairs had photosynthetic activity differences of 5 % or less, while 32 pairs had more than 50 %.

TABLE III

SPECIFIC ACTIVITY OF ASCORBIC ACID FROM APPLE LEAVES AFTER LEAVES HAD PHOTOSYNTHESIZED IN AN ATMOSPHERE OF $C^{14}O_2$

SAMPLE * NO.	LIGHT INTENSITY	TIME	CHANGE IN ASCORBIC ACID CONC	SPEC ACTIVITY OF ASCORBIC ACID
	ft-c	*hrs*	*µgm/disc*	*cpm/100 µgm*
1	600	4	−2	39
2	"	4	0	51
3	"	6	2	47
4	"	6	−1	60
5	"	6	−3	60
6	"	6	−2	31
7	1700	3	0	98
8	"	3	−1	51
9	"	4	1	92
10	"	4	3	119
11	"	4	2	133
12	"	4	0	158
13	"	6	2	196
14	"	6	3	131
15	"	6	2	105

* Each sample consisted of 12 discs, three from each of 4 leaves.

TABLE IV

CHANGES IN THE SPECIFIC ACTIVITY OF ASCORBIC ACID IN APPLE LEAVES SUBJECTED TO VARIOUS TREATMENTS OF LIGHT AND DARK, AT ROOM TEMPERATURE IN ORDINARY ATMOSPHERE

SAMPLE NO.*	SPEC ACTIVITY OF ASCORBIC ACID AFTER 6 HRS AT 1700 FT-C	CONDITIONS OF SUBSEQUENT TREATMENT	SPEC ACTIVITY OF ASCORBIC ACID AFTER SUBSEQUENT TREATMENT
	cpm/100 μgm		*cpm/100 μgm*
1	133	3 hrs dark	140
2	158	"	179
3	105	15 hrs dark	155
4	131	"	151
5	67	3 hrs at 600 ft-c	98
6	31	" "	51
7	28	3 hrs at 1700 ft-c	39
8	30	" "	51
9	31	" "	47
10	35	" "	68
11	92	15 hrs at 1700 ft-c	190
12	119	" "	169

* Each sample consisted of 12 discs, three from each of 4 leaves.

Table IV gives data on the specific activity of ascorbic acid in apple leaves directly after removal from a C^{14} labelled carbon dioxide atmosphere and again after these leaves had been subjected to subsequent different light or dark treatments under normal atmospheric conditions. The incorporation of C^{14} into ascorbic acid continues after removal of the external $C^{14}O_2$ supply. Samples 1 to 12 show that this continued labelling occurs in the light as well as in the dark, indicating that only certain steps of ascorbic synthesis in detached apple leaves are light sensitive.

SUMMARY

In the present study, D-glucuronolactone caused a significant increase in ascorbic acid content of detached apple leaves after 24 hours at 1700 ft-c. After 48 hours no significant increase was found with any of the experimental substances including D-glucuronolactone, L-gulonolactone, D-galacturonic acid, glucose and fructose when compared with the water control. The high correlation coefficient between ascorbic acid content and leaf area indicated the close relationship between photosynthetic activity and ascorbic acid content in apple leaves. The light sensitivity of ascorbic acid synthesis was confirmed by $C^{14}O_2$ experiments but the results suggest that only certain steps in this synthesis are light sensitive.

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STUDIES ON THE PHYSIOLOGY OF TOMATO FRUITS. II. ETHYLENE PRODUCTION AT 20° C AS RELATED TO RESPIRATION, RIPENING, AND DATE OF HARVEST[1]

MILTON WORKMAN[2] AND HARLAN K. PRATT

DEPARTMENT OF VEGETABLE CROPS, UNIVERSITY OF CALIFORNIA, DAVIS, CALIFORNIA

This study establishes the rate and pattern of ethylene production by ripening tomato fruits (*Lycopersicum esculentum* Miller) and relates this to respiration (carbon dioxide evolution) and the stage of ripeness as shown by red color development. The influence of the harvest date has been investigated, since the changing environmental conditions encountered during a harvest season might influence the ethylene producing ability of the fruit and hence its ability to ripen properly late in the season. It has been shown by other workers that the keeping quality of various varieties of apples (9, 12) and pears (8) can be correlated with their ethylene producing ability.

The identification and measurement of ethylene in the emanations of ripening fruits has been considered in recent reviews (1, 13, 15, 16) and in a survey of many subtropical and semitropical species (2). The rate of ethylene production varies among species and among varieties of the same species, both in magnitude and in the nature of the patterns displayed. Biale (1) has stressed the need to search for relations of ethylene production to respiration and storage life of fruits.

The following bioassays have been used to show that tomato fruits produce an active emanation: Stimulation of fruit ripening, epinasty of potato and tomato leaves, "sleepiness" of carnation flowers, and the triple response of etiolated pea seedlings (5, 6, 7, 10, 11, 14). These responses are generally agreed to be due to ethylene production by the fruit tissues. Although direct chemical identification of ethylene as a volatile product of tomatoes has not been reported, selective quantitative methods have been applied. Christensen, Hansen, and Cheldelin (3) used their microbromination method to determine the ethylene content of a ripe tomato fruit and found 0.01 ml/100 gm of tissue. Possible interfering substances were excluded but no precautions were taken against acetylene or propylene since previous work had shown that ethylene was the only unsaturated hydrocarbon present in the volatiles from apples, pears, or bananas. Heinze and Craft (10) used the mercuric perchlorate oxidation method of Gross to follow ethylene production as tomato fruits ripened. From 0.05 to 0.10 mg/kg of fresh weight per day were produced by mature-green fruit at 18.3° C. This rate remained constant for about four days and then gradually increased to 0.40 mg when the fruits were firm-ripe. The relation of ethylene production to respiration has not been studied for the tomato, although this relation has been reported for many other fruits (see reviews cited above).

MATERIALS AND METHODS

In the first paper of this series (17) the following were fully described: Planting dates, harvest dates, harvesting and preparation of samples, method of determining respiration, method of rating fruit ripeness, and the statistical treatment of the data. Two samples of approximately 50 mature-green tomato fruits (Pearson variety) were placed in respiration jars at weekly intervals on six sampling dates in 1952 and seven sampling dates in 1953 for the determination of both carbon dioxide and ethylene production; these pairs of samples are treated in detail in this paper (respiration and ripeness data from these pairs were incorporated with data from other pairs of samples in the previous paper).

Ethylene production was determined by the micromanometric method of Young, Pratt, and Biale (18); ethylene is absorbed from the air stream passing over the tissue by a solution of mercuric perchlorate, forming an ethylene-mercury complex. The accumulated ethylene is later released from the complex by the addition of hydrochloric acid, and its volume is measured manometrically. For measurement of carbon dioxide evolution, the colorimetric method of Claypool and Keefer (4) was used as described previously. In order to measure the rates of carbon dioxide and ethylene evolution simultaneously, a train was arranged as shown in figure 1. This arrangement permitted respiration measurements to be made without inter-

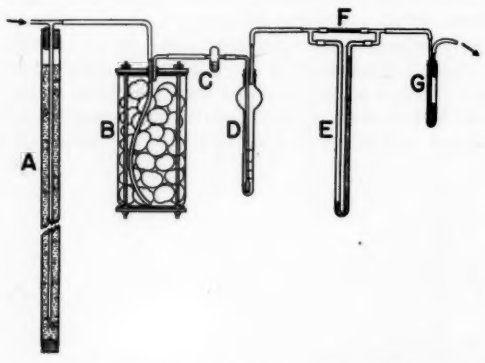

FIG. 1. Arrangements of apparatus for simultaneous determination of ethylene and carbon dioxide production.

[1] Received revised manuscript January 27, 1957.

Based on a portion of a dissertation submitted by the senior author to the Graduate Division, University of California, in partial satisfaction of the requirements for the Ph.D. degree, 1954. This report covers part of a continuing project on tomato fruit ripening in this department.

[2] Present address: Department of Horticulture, Purdue University, Lafayette, Indiana.

rupting the periods of ethylene absorption, since the highly acid mercuric perchlorate solution does not absorb carbon dioxide. The train consisted of a barostat tower (40 mm diameter, 1.6 m long) for pressure regulation (A), the sample container (B), a bulb (C) containing a saturated aqueous solution of n-butyl alcohol, the absorber (D) containing a measured amount of mercuric perchlorate solution and a few drops of n-butyl alcohol and fitted with a fritted-glass dispersion tube (Corning 39533, C porosity), a flowmeter (E) with calibrated capillary (F), and the colorimeter tube assembly (G). The ethylene-absorbing assembly (D) was maintained at approximately 0° C in a vacuum-jacketed ice bath. Ethylene absorption was continuous, and carbon dioxide evolution was measured daily. The latter measurement was made by placing the colorimeter tube assembly in the position shown (fig 1) only during the few minutes required for the carbon dioxide in the air stream to equilibrate with the buffer solution.

After placing the tomatoes in the fruit container, an air flow of known rate (15 to 20 l/hr) was forced continuously through the system in the direction indicated. Regulation of the flow was accomplished by adjusting the bypass tube in the barostat tower so that a pressure of 60 cm of water (the calibration pressure) would be shown at the flowmeter (E). The long barostat was required to provide enough pressure (approximately 120 cm of water) to force air through the fritted-glass bubbler in D and maintain the proper pressure differential across the flowmeter capillary (F). This pressure was maintained on these samples at all times except when the jars were opened to examine the fruit and score it for ripeness. (It was shown in the previous paper (17) that this extra pressure did not seriously affect the rate of respiration or ripening of the tomatoes.) Since the air flow tended to vary because of changes in the porosity of the fritted-glass bubbler, the actual flow was checked and recorded with a calibrated float-type flowmeter before each respiration reading. This also insured against possible error due to partial fouling of the flowmeter capillary (F).

Ethylene absorption periods were usually three days in 1952 and two days in 1953; after each period, the mercuric perchlorate solution was made to volume, removed from the absorber, and replaced with fresh solution. The ethylene evolved by the sample was determined by Warburg manometric measurements on aliquots of the solution. During each absorption period, a volume of air approximately equal in volume to that passed through each sample chamber and from the same source, was passed through another absorber containing mercuric perchlorate as a control on the purity of the air. Any apparent ethylene released by this control solution during the manometric measurement was subtracted from the volume change shown by the parallel samples from the fruit containers; the usual reagent and thermobarometer blanks were also included. The results were calculated as the average rate of ethylene production (in mm^3/kg fruit per day) during the absorption period, and the experimentally determined values were plotted at the midpoint of each period for the graphs and calculations presented below.

TABLE I

RIPENESS SCORING AND DESCRIPTION OF CLASSES USED IN EVALUATING RATE OF RIPENING IN TERMS OF COLOR DEVELOPMENT

SCORE	CLASS NAME	CLASS DESCRIPTION
0	Mature-green	Entirely green, but mature
1	Breaker	First appearance of coloring
2	Light Pink	Approximately equal amount of green and pink
3	Dark Pink	Entirely pink; no green
4	Table Ripe *	Fully red colored; minimum eating ripeness
5	Canning Ripe	Intense red coloring, but still firm
6	Soft Ripe	Perceptible softening (fruit was scored in this class until onset of decay)

* "Table Ripe" was the highest class used in 1952 and ripe fruit was scored in this class until onset of decay.

The ripening rate was observed by removing the fruit from the respiration jars at three-day intervals and sorting each sample into ripeness classes. For convenience, the ripeness scoring method is shown in table I. To determine the average score at each sorting, the number of tomatoes in each class was multiplied by the score assigned to that class, and the summation for all classes was divided by the number of fruits in the sample.

RESULTS

The data for 1953, presented in figure 2, exemplify the work of both seasons. Harvest dates for this year are indicated by the first reading ("0" ripeness) on the individual ripening curves, at which time all fruits were entirely green. Each curve is a smooth line drawn to give the best apparent fit through average respiration, ripening, and ethylene production measurements made on duplicate samples. Since only pairs of samples were available in this study, no statistics of error are shown at the individual experimental points. During these two seasons, only the last harvest of 1952 showed an aberrant pattern of respiration. All other harvests showed the typical climacteric, and all harvests showed a similar pattern of ripening and ethylene production. The general pattern of ethylene production was a rising sigmoid curve; the tomatoes began to produce ethylene in measurable quantities at about the onset of the climacteric rise in respiration which coincides with the first appearance of red color. As the respiration rate increased, ethylene production rose rapidly to a maximum, continuing its rise after the climacteric was passed and respiration markedly decreased. Occasionally there was a slight drop toward the end of the experiment.

Figure 3 presents seasonal summaries for the years

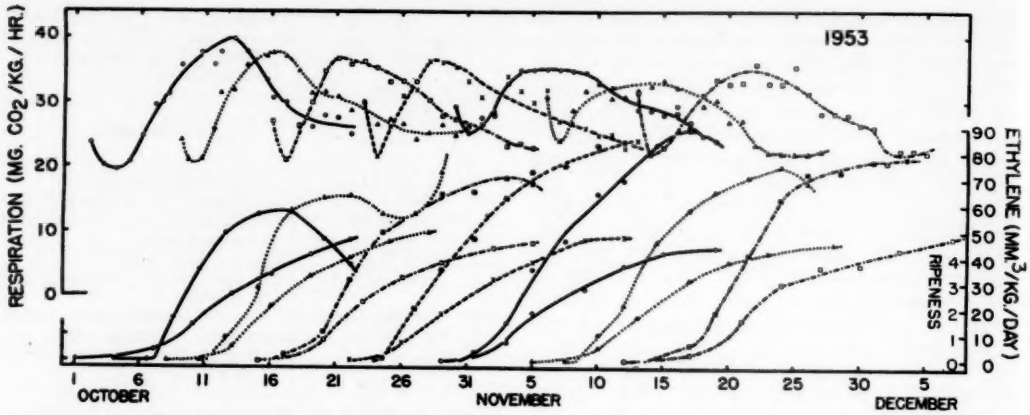

FIG. 2. Respiration, ripening, and ethylene production patterns for 1953 harvests. Each point is the mean of two samples. Curves for a given harvest have corresponding symbols, with ethylene production indicated by solid symbols and the date of harvest indicated by the initial ripeness score of "0".

1952 and 1953; for each season the data were summarized by taking the average readings for each individual harvest and calculating the mean of the means. In other words, in figure 3 (1953), all data of figure 2 are averaged on the basis of days after harvest. Almost without exception, respiration readings were taken daily, and the summaries of respiration data used actual numerical values obtained on that particular day (to the nearest day). On the other hand, because ripeness scores for different harvests were not always taken the same number of days after harvest, the seasonal ripeness summaries are made up of readings from smooth curves drawn through each set of data for the respective individual records (as shown in fig 2). In 1953, ethylene readings were taken at the same intervals after harvest throughout the season, and the summary curve is made from these values; for 1952, the rates of ethylene production were read from the individual harvest curves. Vertical lines drawn through the experimental points of the summary graphs of figure 3 indicate the standard error of the mean of the individual harvest mean values used in the summary calculation, the odds being two to one that data obtained from paired samples on several harvest dates during the fall season would give the same results within these limits in the respective years.

Some differences may be observed between the average respiratory, ripening, and the ethylene production patterns obtained in the two years. In general, the rate of respiration and the rate of ethylene production were higher in 1952 than in 1953. However, experimental technique was improved in the 1953 season, and this may account for a part of the difference. The ripeness scores indicated in figure 3 are not directly comparable between the two years because two more classifications were used in 1953; hence the curve for ripeness in 1953 seems to rise higher than 1952. In 1952 the maximum of carbon

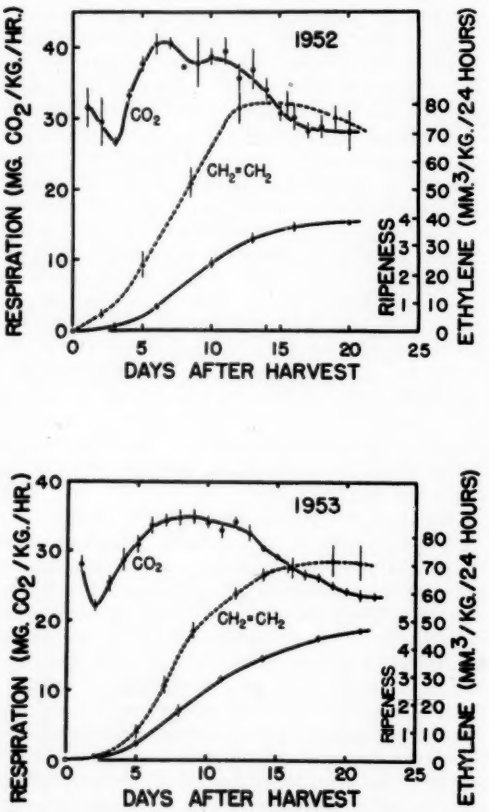

FIG. 3. Seasonal averages of mean rates of respiration and ethylene production and of ripeness scores, based on six harvests for 1952 and seven for 1953. Standard errors of the means are indicated by vertical lines.

dioxide production was reached earlier than in 1953, and the rate at the peak was higher; the pre-climacteric minimum was later in 1952 than in 1953, and the minimum was higher. In 1952 ethylene production reached its maximum earlier than in 1953 and a higher maximum production was reached. In 1952, at the time the climacteric peak of respiration was reached, ripeness of the sample averaged between breaker and light pink. At the time maximum ethylene production was reached, the sample averaged between "dark pink" and "table ripe." In 1953 at the time the peak of respiration was reached the sample averaged between "light pink" and "dark pink" in ripeness and at the time the maximum of ethylene production was reached the sample was "table ripe."

Discussion

In earlier discussion (17) various aspects of tomato ripening and respiration rate have been considered in relation to field weather conditions during the growing seasons studied. These remarks, based on four samples per harvest, apply equally well to a consideration of that pair of those four treated herein. A study of the behavior of the individual harvests of both years failed to reveal any consistent seasonal changes in the ability of the tomatoes to produce ethylene, in spite of the slower ripening and some changes in respiration rates that have been shown. Even though the last harvests of both 1952 and 1953 were subject to some chilling injury, no difference in the pattern of ethylene production was observed. It appears, therefore, that the ethylene producing ability of the fruit is not directly related to its ability to ripen properly after harvest. At least, it must be assumed that the amount of ethylene produced is more than sufficient to fulfill any role which ethylene may play.

The pattern of ethylene production by Pearson tomatoes was found to be similar to that of the Rutgers variety grown in Maryland as shown by Heinze and Craft (10), but the rates they reported are very much higher. They show an appreciable production of ethylene while the fruit is still green, whereas with Pearson fruits no ethylene was found at this stage. Denny (5) reported an active emanation from immature tomatoes, but his fruits were chopped and the ethylene production might have been an injury response. Some variation is to be expected between varieties; in one experiment in this laboratory, a seedling line from breeding stock gave a much higher rate of ethylene production than did Pearson while showing a similar rate of carbon dioxide production.

Information on the relationship of ethylene production and respiration has not been available for the tomato. In other fruits (2) the respiratory climacteric may coincide with a peak in ethylene production, or an ethylene peak may follow the maximum of carbon dioxide production. The tomato appears to be unique; none of the fruits previously studied have shown a high rate of production of ethylene maintained to the end of the experiment when the fruits are full-ripe to over-ripe. The completion of ripening in tomato is more closely associated with the ethylene maximum than with that of carbon dioxide.

Table II

Ratios of Ethylene Production to Carbon Dioxide Production *

Ratios	1952	1953
Maximum ethylene rate/maximum CO_2 rate	0.165	0.170
Maximum ethylene rate/CO_2 rate at same time	0.220	0.238
Ethylene rate at CO_2 max/max CO_2 rate	0.082	0.110

* Manner of calculation: $\frac{mm^3 \text{ ethylene} \times kg^{-1} \times hr^{-1} \times 1000}{ml\ CO_2 \times kg^{-1} \times hr^{-1}}$

Biale et al (2), have shown that low rates of carbon dioxide production are generally associated with low or undetectable rates of ethylene production, and have tabulated ethylene to carbon dioxide ratios for a number of fruits. The tomato has a relatively low rate of respiration and an especially low rate of ethylene production; the latter gives it one of the lowest ratios of ethylene to carbon dioxide production of any fruit. Table II presents some aspects of these relations drawn from the summary graphs of figure 3. Biale et al (2), calculated their ratios on the basis of the maximum rates of production of each gas, whether or not the maxima coincided in time. This approach seems valid only for those fruits where the production patterns for both gases are similar and the maxima do indeed coincide; as table II shows, ethylene/carbon dioxide ratios may vary as much as 270 %, depending on an arbitrary choice among several legitimate possibilities. The lack of direct correlation between ethylene production and carbon dioxide production is obvious.

Summary

The pattern of ethylene production by ripening tomato fruits was established in relation to respiration and the rate of ripening. The general shape of the ethylene evolution pattern was found to be that of a sigmoid curve. Production of ethylene in measurable quantities began with the first appearance of color and at about the onset of the climacteric rise in respiration. A definite relationship between the maximum rates of ethylene evolution and of carbon dioxide production was not observed. Many fruits show a peak of ethylene production, but tomatoes appear to produce the most ethylene near the end of the storage life. The total amount of ethylene produced by tomatoes during ripening is very small compared to other fruits, and their ethylene/carbon dioxide ratio is consequently one of the lowest. No consistent seasonal changes in the pattern or magnitude of ethylene production were observed; hence, it appears that the failure of mature-green tomatoes to ripen properly

late in the season is not related to their loss of ability to produce ethylene.

We are indebted to Professor Leonard L. Morris for advice and to Mr. Carl L. Tucker for technical assistance.

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CHANGES IN THE INOSITOL CONTENT DURING GERMINATION AND GROWTH OF SOME HIGHER PLANTS[1,2]

KEITH E. RICHARDSON[3] and BERNARD AXELROD

Department of Biochemistry, Purdue University, West Lafayette, Indiana

Since its initial discovery by Scherer (1) in 1850, mesoinositol has been found in a great variety of plants and animals. It has been shown to exist in the free state and in combined forms as, for example, phytin, phosphoinositides, galactoinositides, etc. Because of its widespread occurrence inositol has attracted the attention of numerous investigators and has been the subject of extensive research. Nonetheless, while Magasanik (2) has established the degradative pathway for microbial utilization of inositol, little information is available concerning its metabolism in higher plants. The only creditable experimental evidence bearing on the metabolic pathway of inositol in higher plants that has been put forth is in the reports of Kursanov et al (3). These workers infiltrated glucose and also inositol into tea leaves. From analyses performed on the leaves after suitable periods of incubation, they concluded that inositol was an intermediate in the conversion of glucose to phloroglucinol. These findings apparently have not yet been confirmed in other laboratories.

In an effort to locate tissue in which there is a rapid metabolism of inositol for the study of reactions concerned with formation, utilization, and breakdown of inositol we undertook an investigation of the change in content of free and bound inositol in germinating seeds and seedlings. Recently Darbree and Norris (4) have followed the free and bound inositol in germinating oats for a period of 10 days and have reported an initial decrease. We wish to report related studies made with a number of other species for a somewhat more extended period of time. We have also studied the fixation of $C^{14}O_2$ into inositol of seedlings.

The initial decrease noted in total inositol in the case of all seeds tested in the present work agrees with the findings of Darbree and Norris for germinating oats and indicates that such an initial decrease

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is general among higher plants. This is in contradiction to the work of Burkholder (5) and Burkholder and McVeigh (6) who reported an initial increase of inositol in germinating oats, mung beans, peas, and soybeans.

Materials and Methods

The following seeds were used: Pea (*Pisum sativum* L., var. Alaska), mung bean (*Phaseolus aureus* Roxb.), soybean (*Glycine Max* (L.) Merr., var. Hawkeye), corn (*Zea Mays* L., var. 813 US 13 hybrid), wheat (*Triticum aestivum* L., var. Knox), and cabbage (*Brassica oleracea* L.).

Germination: For light germination studies the seeds were planted in sand in two-inch-deep waxed cardboard containers and germinated at 25° C in continuous fluorescent light. Every fourth day the seeds were watered with a mineral solution (1 gm $Ca(NO_3)_2 \cdot 4\,H_2O$, 0.25 gm KNO_3, 0.25 gm KH_2PO_4, and 0.25 gm $MgSO \cdot 7\,H_2O$ in 1 liter of water). In dark germination studies the seeds were germinated and permitted to develop on moist filter paper at 25° C. The seedlings were removed at the specified intervals, washed thoroughly to remove all traces of sand, and dried at 87° C to constant weight.

Determination of Inositol: The dried samples were ground in a micro-Wiley mill fitted with a 40-mesh sieve. The free inositol of a weighed sample (approximately 0.5 gm) was extracted by refluxing for 24 hours with 50 ml of 80 % (v/v) ethanol. After evaporation to dryness under vacuum the sample was taken up in water, decolorized with charcoal, filtered, and diluted to a volume of 50 ml. The clear solution obtained was then assayed for inositol. Bound inositol was converted to free inositol by the acid hydrolysis method of Woolley (7). The biological assay method of Atkin et al (8) utilizing *Saccharomyces carlsbergensis* was used to determine free inositol. This method is generally accurate to ± 10 to 15 %.

Chromatography: The following solvents were used in the chromatography of inositol: butanol–ethanol–water, 10 : 1 : 2 (9); butanol–acetic acid–water, 5 : 1 : 2 (10); butanol–pyridine–water, 4 : 1 : 1 (11); butanol–diethylene glycol–water, 3 : 1 : 1 (12) and pyridine–amyl alcohol–water, 14 : 14 : 12 (13). All proportions are on volume basis. The position of the inositol was found by using the alkaline $AgNO_3$ dipping reagent of Trevelyan et al (14). Where the inositol was radioactive it was located by using x-ray film.

Formation of Radioactive Inositol: The plant material was placed in a $C^{14}O_2$ atmosphere (approximately 200 μc) for five days under continuous light. At the end of this period the plant material was removed, sliced, and the soluble carbohydrates extracted with 80 % ethanol in a Soxhlet extractor for 24 hours. The resulting solutions were evaporated to dryness, redissolved in water, decolorized with charcoal, and deionized with Dowex 50 and Amberlite IRA 400. The clear solutions were concentrated in air and spread in bands on Whatman No. 1 filter paper and chromatographed using butanol–ethanol–water. Reference spots of unlabeled inositol were placed at the edge of the band to assist in the location of the labeled inositol. Bands corresponding to inositol were located with x-ray film, eluted with water, and concentrated. The samples were spotted on Whatman No. 1 filter paper and chromatographed using five different solvents. In each case activity appeared only in the inositol area.

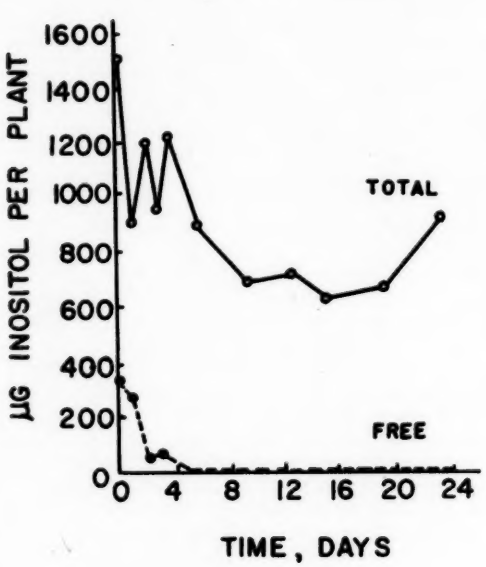
Results

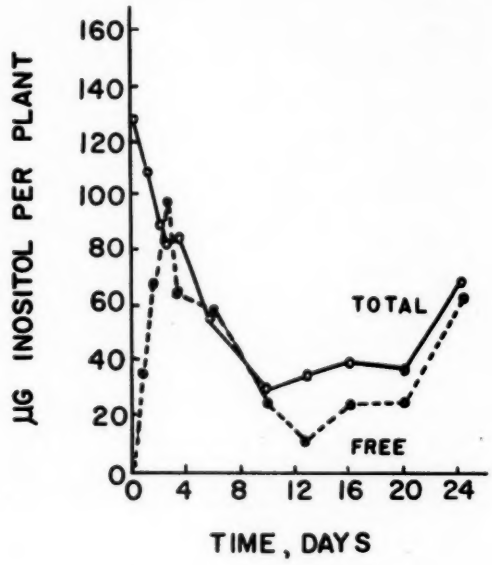
In all cases reported here there was an initial decline in total inositol content per plant on germination. This was equally true for peas, mung beans, cabbage and corn grown in the light (figs 1 to 4), and for peas and mung beans grown in the dark (figs 5 and 6). Wheat and soybeans also suffered a decrease in inositol content when grown in the light. The decline was ultimately replaced by an increase as the plants grown in the light continued their development. It is interesting to observe the extremes in the ratio of free to bound inositol which can arise in different species. Peas (fig 1) after a few days had only a small portion of their inositol in the free form whereas mung beans (fig 2) at the same period of growth had but a small fraction of their inositol in the bound form. The total inositol contents of the peas and the mung beans were, respectively, 8650 and 3600 μgm per gm dry weight of the seed. After 4 days of growth, the corresponding values were 10,250 and 4020 μgm per gm. This increase in concentration on a dry weight basis, while the quantity per seedling decreases is a consequence of loss of dry weight during germination.

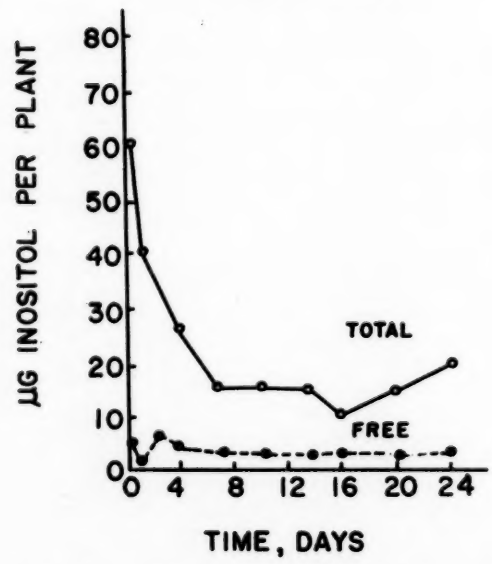
In view of the possibility that this loss of inositol from the plant might be due to diffusion out of the plant into the growth media, acid washed sand in which 100 peas were germinated for 10 days was assayed for inositol. A value of 2 μgm of inositol per seed was obtained from the sand while the total loss in 10 days of germination was 600 μgm per seed showing that the loss of inositol by this means is insignificant. It would thus appear that inositol is metabolized by the germinating seeds.

Although the 10-day-old etiolated peas were undergoing a net decline in total inositol, just as were those grown in the light, they proved capable of synthesizing inositol on exposure to light and $C^{14}O_2$. Formation of inositol from $C^{14}O_2$ in the dark was not studied. With these seedlings, the quantity of labeled free inositol produced was small as might have been predicted from the very low content of free inositol which occurs in pea seedlings shortly after germination. The radioactive inositol so obtained was identical with authentic inositol in its chromatographic behavior in all of the solvent systems used. However, it was felt desirable to prove its identity by the more rigorous criterion of crystallization to constant specific radioactivity. Unfortunately, the small quantity of material precluded an accurate experi-

ment of this type. For this reason radioactive inositol was prepared in sufficient amounts for this purpose using pea tissue sufficiently mature to perform good synthesis, namely the maturing pea pod. The pod was approximately 5 cm in length and contained seeds about 3 mm in diameter. The pod was left attached to a small section of stem which was placed in a small beaker of H_2O. The inositol produced by the pod was isolated in the manner described above and mixed with authentic carrier inositol. The relative specific activities in counts per min per mg after five successive crystallizations from aqueous ethanol were for the first through the fifth crystallizations, respectively: 16,920; 17,390; 16,800; 17,300 and 17,400. Thus it seems unlikely that the radioactive "inositol" formed by the seedlings could have been due to some







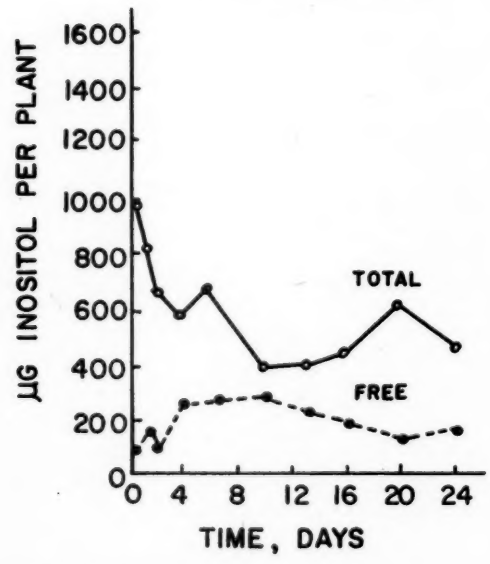
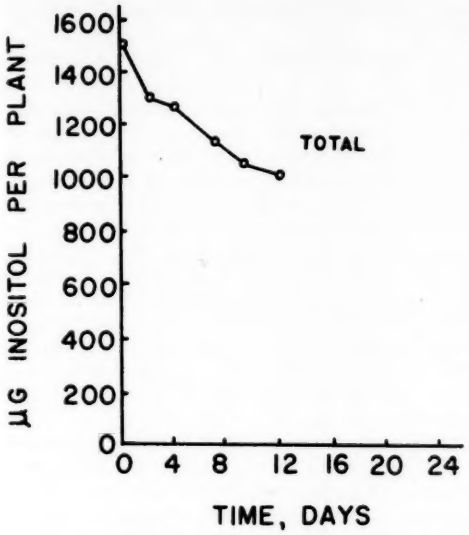


FIG. 1 (*upper, left*). Total and free inositol content of light-grown pea seedlings during development.
FIG. 2 (*upper, right*). Total and free inositol content of light-grown mung bean seedlings during development.
FIG. 3 (*lower, left*). Total and free inositol content of light-grown cabbage seedlings during development.
FIG. 4 (*lower, right*). Total and free inositol content of light-grown Zea seedlings during development.



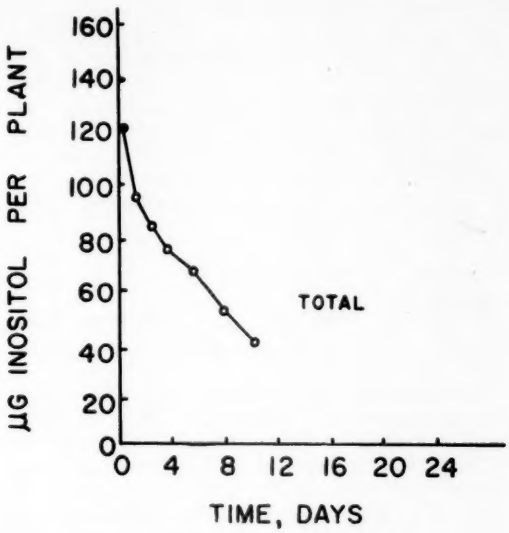


FIG. 5 (*left*). Total inositol content of dark-grown pea seedlings during development.
FIG. 6 (*right*). Total inositol content of dark-grown mung bean seedlings during development.

unknown radioactive compound with precisely the same mobility as inositol.

SUMMARY

1. Six different species of plants (cabbage, corn, mung bean, pea, soybean and wheat) all showed an initial decrease in their total inositol content on being germinated and grown in the light. A similar decrease was observed with etiolated peas and mung beans.

2. The decrease did not appear to be due to loss of inositol by diffusion out of the roots.

3. The initial decrease in total inositol content was followed by an increase as the plants underwent further development in the light.

4. The 10-day-old etiolated pea seedling as well as the immature pea pod can incorporate $C^{14}O_2$ into free inositol in the presence of light. Incorporation in the dark was not studied.

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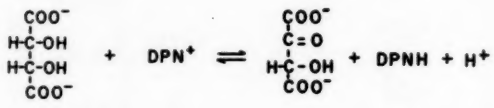
TARTARIC ACID DEHYDROGENASE ACTIVITY IN HIGHER PLANTS[1,2]

HELEN A. STAFFORD

BIOLOGY DEPARTMENT, REED COLLEGE, PORTLAND, OREGON

An enzyme which catalyzes the oxidation of (−)- and meso-tartrate to the enol or keto form of dihydroxyfumarate (DHF) by a diphosphopyridine nucleotide (DPN) dehydrogenase can be demonstrated in higher plants. The reaction is postulated as follows:



Although the tartrates have been considered as possible metabolites in higher plants since the initial work of Pasteur in 1848, surprisingly little is known of their actual physiological significance. Vickery and Palmer (35) have briefly reviewed the history of (+)-tartrate in plants, including the problems of identification and of nomenclature. They conclude that (+)-tartrate is an uncommon plant acid produced from glucose by a few plants like grapes. In their own experimental work with tobacco leaves, these authors report that the (+)-isomer is metabolically inert, and that it has no effect on the production of the enzymatically active L-malic acid. No reference is made, however, to either the meso- or the (−)-forms as possible metabolites, the latter isomer having the configuration of the enzymatically active L-malic acid. The meso-form could have been overlooked in plants because it has solubility characteristics which differ from those of the other tartrates.

Bacteria and molds, on the other hand, can oxidize both the (−)- and (+)-isomers of tartaric acid, the (+)-isomer being the most active (34). Recently, bacterial extracts have been reported capable of converting either (−)- or (+)-tartrate to oxalacetate (17, 20), and tartrate is postulated as an intermediate in the production of oxalate from oxalacetate in *Aspergillus niger* (2).

Furthermore, the (−)- and meso-isomers of tartrate are active metabolites in extracts of animal tissues. Scholefield (28) investigated a DPN-linked dehydrogenase in pigeon liver which oxidizes meso-tartrate, while Kun et al (15, 16) reported a mitochondrial dehydrogenase in rat and beef tissue which is capable of oxidizing both the (−)- and meso-forms, but not the (+)-isomer.

The presumed oxidation product, dihydroxyfumarate (DHF) has been reported in higher plants (7, 18, 27), but the instability of this compound would make its identification very difficult. Furthermore, the presence of DHF could easily be confused with ascorbic acid because of similar chemical properties (7).

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[2] This investigation was supported by a research grant from the National Science Foundation (NSF-G 1277).

The chemistry of DHF and its non-enzymatic oxidation and decarboxylation products was first reported by Fenton in a series of articles in which the following breakdown compounds were discussed: glycolaldehyde, diketosuccinic, ketomalonic (mesoxalic), hydroxymalonic (tartronic) acids, mesoxalic-semialdehyde, glyoxal, and the 3 isomers; tartronic-semialdehyde, dihydroxyacrylic and hydroxypyruvic acids (3 to 6). Later, oxalic and glyoxylic acids were added to this list of compounds (19). These side reactions of DHF, which make this compound a difficult one to use experimentally, will be discussed later along with the possible role of DHF in plant metabolism (fig 3).

The present work on tartaric acid dehydrogenase in higher plants was started as a result of investigations concerning diketosuccinic acid reductase and hydroxymalonic acid dehydrogenase (30, 32). The activity of (−)-tartrate was briefly reported in the latter paper, and a preliminary account of the present data has already been made (33).

MATERIALS AND METHODS

Enzyme preparations from peas (*Pisum sativum*), wheat germ S-50, and beans (*Phaseolus vulgaris*) were made from acetone powders with subsequent precipitation with ammonium sulfate as previously described (32), except that treatment with $MnCl_2$ was omitted. The fraction used was that obtained between 200 to 400 gm ammonium sulfate per liter of enzyme solution. Typical protein content (32) of the major preparations used was as follows: wheat germ, 46 mg per ml extract; 12-day-old green shoots, 8.2 mg per ml; pea seeds soaked for 12 hours, 7.9 mg per ml.

The tartrates were recrystallized as potassium acid salts (32); (+)-tartaric acid was obtained from Merck (reagent grade), (−)-tartaric (labelled L-) and meso-tartaric acids were purchased from both Aldrich Chemical Company and Nutritional Biochemicals Corporation. Samples of DHF used were either made from (+)-tartaric acid or were purchased from Aldrich Chemical Company and were recrystallized according to Hartree (8). The purity of the samples (96 to 100 %) was determined spectrophotometrically by light absorption of the enol form at 292 $m\mu$ (8) and by a $K_3Fe(CN)_6$—Prussian blue analysis for both enol and keto forms (21). The DPNH used was purchased from Sigma Chemical Company. Glycoaldehyde was made from DHF according to Fenton (4). After treatment with Dowex 1 ($CO_3^=$ form) to remove any free acid, analysis with crystalline alcohol dehydrogenase indicated a minimum purity of 85 to 90 %. Hydroxypyruvate of 95 % purity by enzymatic analysis was made from methylbromopyruvate (Aldrich Chemical Co.). Other compounds used were described in previous papers (30, 31, 32).

The tartrates were analyzed by the naphthoresorcinol (32) or sodium vanadate methods (34), the latter being adaptable for paper chromatography for the optically active isomers. Reduced DPN (DPNH), hydroxymalonate, and DHF were determined by the $K_3Fe(CN)_6$—Prussian blue analysis. Hydroxymalonate, DHF, and hydroxypyruvate were analyzed by the molybdate method (32). The 2,4-dinitrophenylhydrazine colorimetric and chromatographic methods used were described previously (32).

Experimental Results and Discussion

Demonstration of the Aerobic Oxidation of Tartrate to DHF by Spectrophotometric Observation of the Reduction of DPN: The reduction of DPN in the presence of added tartaric acid, indicated by an increase in absorption of 340 mμ, is shown in figure 1. The curves illustrate the difference in activity between meso- and (−)-tartrate in wheat germ preparations. Comparable results were obtained with pea enzyme preparations. No measurable activity is observed in the presence of the (+)-isomer. The final equilibrium values with either of the active tartrates vary somewhat with different preparations, probably because of variations in side reactions which affect the products of the reaction. Under the conditions, described in figure 1, the enzyme was saturated with

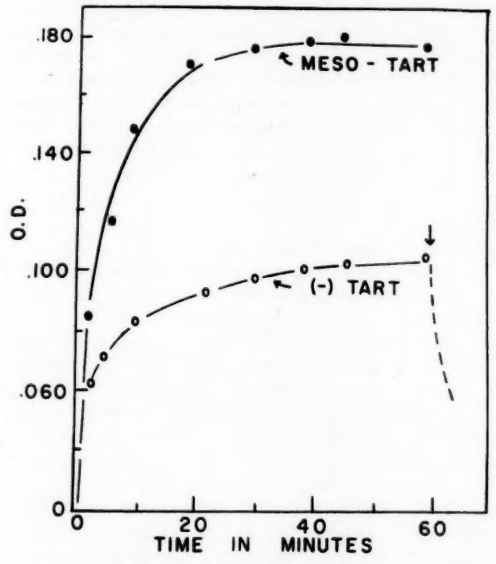

Fig. 1. Reduction of DPN by tartrate in the presence of wheat germ enzymes. The light absorption (O.D.) at 340 mμ due to DPNH is plotted against time. Cuvettes contained the following in a total volume of 3 ml: 0.5 ml of 0.5 M TRIS buffer (pH 9.0), 0.4 mg DPN^+, 100 micromoles of meso- or (−)-tartrate, and 9.2 mg protein from wheat germ extract. Blank values of DPN reduction without added substrate have been subtracted.

Table I

Anaerobic Oxidation of Tartrate in the Presence of $K_3Fe(CN)_6$

Hrs incubation	Tartrate	Micromoles $K_4Fe(CN)_6$ produced *	Micromoles DPNH or DHF calculated **
1	Meso-	1.71	(0.43)
2	Meso-	2.92	(0.73)
4	Meso-	5.26	(1.31)
2	(−)-	0.69	(0.17)
2	(−)- + Mg^{++}	0.59	(0.15)
2	Meso-	2.52	(0.63)

Thunberg tubes contained the following in a 3 ml volume: 0.5 ml 0.5 M TRIS (tris-hydroxymethyl-aminomethane), 0.2 mg DPN^+, 23 mg wheat germ protein, 200 micromoles tartrate, 150 micromoles Mg^{++}, 60 micromoles $K_3Fe(CN)_6$. Blank values have been subtracted.

* Prussian blue analysis.

** Calculated from above analysis.

100 micromoles of substrate, and extra DPN did not increase the rate. When 150 micromoles of acetaldehyde were added to the cuvette with (−)-tartrate at 60 minutes, there was a rapid decrease in absorption at 340 mμ as the DPNH was oxidized by the alcohol dehydrogenase present in the enzyme mixture. Unlike the enzyme reported by Kun (15, 16), there was no noticeable effect of either added Mg^{++} or ethylenediamine-tetraacetic acid in these plant preparations. Whether this is due to a difference in the activity of the enzyme itself, or to the presence of substances or side reactions that would obscure this effect has not been determined. Furthermore, the plant activity is a far more sluggish one than that reported for animal tissues. The presence of hydroxylamine to trap the DHF formed doubled the rate of the reduction of DPN. Since the sodium salts of tartrate appeared to be less active than the potassium salts, KOH was used to neutralize the tartaric acid in the work reported here. Anaerobic experiments, performed in evacuated Thunberg tubes, appeared to have no effect on the final amount of DPN reduced. TPN is inactive even when 10× the amount of DPN used was added.

Demonstration of the Anaerobic Oxidation of Tartrate in a Coupled System: While neotetrazolium and 2,6-dichlorophenolindophenol were reduced anaerobically in the presence of tartrate via diaphorase, the use of $K_3Fe(CN)_6$ as the ultimate electron acceptor was the most sensitive method used. DPNH and hydroxymalonate in trichloracetic acid filtrates give a positive test when using the Prussian blue colorimetric method to determine the $K_4Fe(CN)_6$ formed either before or after the addition of the Prussian blue reagent. (The Prussian blue color produced by hydroxymalonate is characterized by a 2- to 3-minute lag in the appearance of the color after the reagents are added.) Although both DHF and hydroxymalonate react in this test, and the values are additive when the substances are analyzed together, only a one step oxidation is observed when DHF is

the starting substrate. This indicates that the presumed oxidation product, diketosuccinate, does not decarboxylate to hydroxymalonate under the conditions of the test. Attempts to analyze the products of this $K_3Fe(CN)_6$ oxidation were not successful.

Typical results are shown in table I for a wheat germ preparation. Similar results were obtained with a pea leaf enzyme preparation. The reaction is roughly linear with time for about a 4-hour period. Assuming that both DPNH and DHF give only a one step oxidation each, these $K_4Fe(CN)_6$ values can be divided by 4 to give the amount of DPNH or of DHF produced. Again the meso- form of tartrate is more active than the (−)- form and added M_g^{++} did not alter the results appreciably.

Identification of the Reaction Products of the Aerobic Oxidation of Tartrate: The immediate reaction product of tartrate oxidation is presumably DHF. If the enol rather than the keto form of DHF were the first oxidation product, an increase in absorption would be expected at 292 mμ where the enol form absorbs strongly. Under aerobic conditions, such an increase does not occur. This indicates either that the enol form is absent or that ketonization occurs too rapidly.

An indication of the formation of DHF (enol or keto form) as the major reaction product is shown in table II. In these aerobic tests with meso-tartrate as substrate, the reaction was followed spectrophotometrically at 340 mμ for 1 to 3 hours. The protein was precipitated with trichloracetic acid (3 % final concentration), and molybdate, $K_3Fe(CN)_6$—Prussian blue and total hydrazone analyses were made on unheated and heated aliquots (held in a hot water bath at 80° C for 30 minutes). Sample data are shown in table II for different enzyme preparations. If any of the DHF formed is converted to glycolaldehyde, this would not be accounted for in the values of this chart except in the amount of DPN reduced. Many of these preparations also contain active DPNH oxidase or DPN destroying activities which would tend to lessen the apparent amount of DPN reduced. Low $K_4Fe(CN)_6$ values in comparison with the value expected from the amount of DPNH formed according to the absorption at 340 mμ could be attributed to the side reaction to glycoaldehyde. This tends to be more evident with the longer incubation periods. Any hydroxypyruvate formed would decarboxylate to glycoaldehyde above pH 8.5. Although the $K_3Fe(CN)_6$-Prussian blue analysis on unheated aliquots would detect DHF, DPNH and hydroxymalonate, only the latter would be detectable in the heated aliquots. These data indicate some of the variation found. For example, hydroxymalonate can be detected with long incubation periods in some preparations, but not in others. An increase in total hydrazones can be demonstrated in separate tests. Data are shown here for a pea seed supernatant fraction, but similar increases in total hydrazones were observed in particulate preparations also. No evidence is shown here for the second possible step; i.e, DHF to diketosuccinate. If this occurred, larger values for hydroxymalonate would be expected.

Analyses of the 2,4-dinitrophenylhydrazones formed by methods previously described (32) and by those of Kun (15), were not considered accurate enough for quantitative results. Even qualitative identification of these derivatives is difficult because a variety of osazones as well as hydrazones, some of which can exist as cis-trans isomers, have been reported as de-

Table II

Aerobic Oxidation of Meso-Tartrate by Plant Enzymes

Enzyme source	Mg protein used	Min incubation	Micromoles produced					
			DPNH *	$K_4Fe(CN)_6$**	DHF + OHM		OHM ‡	DHF ‡‡
					Calculated †	Mo test ††		
Wheat germ	9.2	60	0.078	0.322	(0.081)	....	...	...
		75	0.043	0.230	(0.058)	0.062	...	...
Pea seed	1.6	60	0.045	0.176	(0.044)	....	0[a]	...
		150	0.05	....	....	....	...	0.04
		180	0.067	0.214	(0.053)	....	0[a]	...
		180	0.074	0.100	(0.025)	....	0.03	...
Pea shoot	3.0	90	0.051	0.258	(0.065)	....	...	...
	0.8	180	0.085	0.220	(0.055)	....	...	...

Cuvettes contained in a total volume of 3 ml: 0.5 ml TRIS buffer (pH 9.0), 0.4 mg DPN+, 200 micromoles of substrate, plus enzyme. Blank values were subtracted.

* ΔO.D. at 340 mμ.

** Prussian blue test on unheated aliquots.

† Calculated from above value (divided by 4).

†† Molybdate test on unheated aliquots.

‡ Prussian blue test on heated aliquots.

‡‡ Total 2,4-dinitrophenylhydrazone value on unheated aliquots.

[a] Not detectable with largest aliquot possible.

rivatives of DHF (6, 9, 29). Tentative evidence of the formation of 2,4-dinitrophenylhydrazone derivatives of DHF, diketosuccinate, and of glycolaldehyde in extracts of these enzyme mixtures was obtained chromatographically, but the author feels that a better understanding of DHF and its decomposition products is required before more definitive qualitative and quantitative evidence can be obtained.

Demonstration of the Oxidation of DPNH by DHF: The spectrophotometric demonstration of the reduction of DHF to tartrate via DPNH oxidation is complicated by the presence of at least two other reactions occurring in the plant extracts used which result in products which can subsequently oxidize DPNH. These are the autoxidation of DHF to diketosuccinate, and the non-enzymatic decarboxylation of the DHF to glycolaldehyde. Other possibilities are the production of hydroxypyruvate or glyoxylate, which likewise could be involved in further oxidation of the DPNH.

The graphs shown in figure 2 and those reported previously, indicate that DPNH is oxidized both aerobically and anaerobically upon the addition of DHF. The reaction which occurs aerobically probably involves the autoxidation of diketosuccinate and the subsequent oxidation of the DPNH by the diketosuccinic acid reductase activity (30). Kun (16) reports no difficulty with this side reaction and performs his spectrophotometric tests aerobically. Activity curves are shown here for wheat germ and pea shoot enzyme preparations. Curves for pea seed preparations would be comparable to those shown for wheat germ. Unfortunately, only small amounts (about 0.2 micromole) of DHF can be added; otherwise, there would be some absorption at 340 mμ due to the enol form. Anaerobic oxidation does occur in these preparations, but only at a relatively slow rate in comparison with the aerobic activity with DHF or with diketosuccinic acid reductase activity. The curve for activity with diketosuccinate is that observed when 1 micromole of substrate is added; comparable initial rates are obtained with 0.2 micromoles of substrate, but there is an indication of a decline in reaction rate with time. Starting with 0.2 micromoles of DHF in the presence of wheat germ enzyme (containing 2.3 mg protein per cuvette), about 0.02 micromoles of DPNH were oxidized in 15 minutes anaerobically, and about 0.13 micromoles after 2 hours of incubation anaerobically. This can be compared with a value of about 0.062 micromoles after 2 hours incubation with a pea shoot preparation (containing 1.7 mg protein). While these slow rates in contrast with the reductase activities of ketomalonate and of diketosuccinate could be due to a sluggish enzyme system, the inability to add an excess of substrate and the multiplicity of reactions occurring could also account for the slowness of the rate.

The remaining curves in figure 2 indicate the possible cause(s) of this anaerobic oxidation of DPNH. In pea shoot preparations, the oxidation of DPNH appears to be due to the reversal of tartrate dehydrogenase activity, while in the presence of wheat germ preparations the oxidation could be attributed to the combined activities of this same tartrate dehydrogenase plus alcohol dehydrogenase which would reduce the decarboxylation product glycolaldehyde to ethylene glycol.

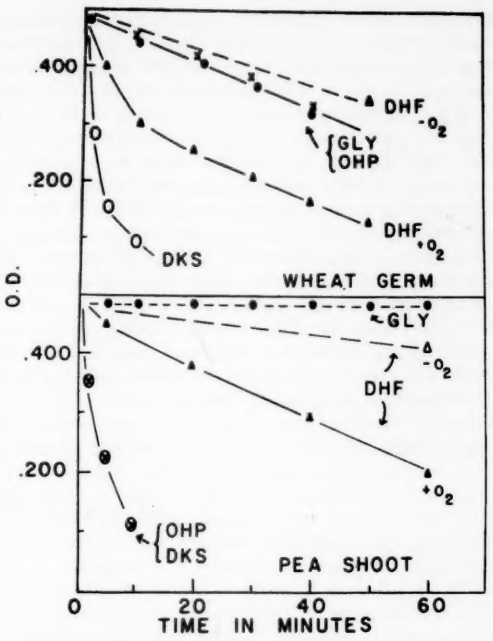

Fig. 2. Oxidation of DPNH in wheat germ and pea shoot enzyme preparations in the presence of different substrates. The light absorption (O.D.) at 340 mμ due to DPNH is plotted against time. The cuvettes contained in a total volume of 3 ml: 0.2 ml of 0.5 M phosphate or TRIS buffer (pH 7.4), 0.25 micromoles DPNH, 0.2 micromoles DHF, 1 micromole diketosuccinate (DKS), 4 micromoles glycolaldehyde (GLY) and hydroxypyruvate (OHP), and 2.3 mg protein from wheat germ or 1.7 mg from pea shoot enzyme preparations. All experiments were made aerobically, except in the case of one of the curves for DHF (open triangles) which were performed anaerobically (30). Blank values of DPNH oxidation without added substrate have been subtracted.

The following data suggest that neither hydroxypyruvate nor glyoxylate are formed in significant amounts. Leaves contain active glyoxylic and hydroxypyruvic acid reductases in contrast to seeds (13). If significant amounts of either glyoxylate or hydroxypyruvate were produced from DHF, one might expect to find very different rates of DPNH oxidation with the two different types of preparations. Figure 2 shows DPNH oxidation curves obtained in the presence of hydroxypyruvate in the two preparations. Similar curves would be obtained with

4 micromoles of glyoxylate, and both enzymes are quite active even with trace amounts of substrate. The anaerobic activity shown for DHF with pea shoot enzymes, therefore, would not be due to the formation of either hydroxypyruvate or glyoxylate as has been reported by Kun (15, 16). If one assumes that the wheat germ has no unique effect on the decarboxylation of DHF, the oxidation of DPNH in such preparations would likewise not be due to hydroxypyruvate, although at this high enzyme concentration there is an oxidation with hydroxypyruvate comparable to that obtained with added glycolaldehyde.

Data were obtained, however, which indicate the slow but steady production of glycolaldehyde aerobically and anaerobically in the presence and in the absence of wheat germ and pea shoot preparations. The first evidence was the production of CO_2 when either of these enzyme preparations were incubated anaerobically (N_2 gas) in Warburg vessels. Starting with 7 micromoles of DHF, and 7 mg protein from a pea shoot preparation, the evolution of CO_2 continued for a period of over two hours, with approximately 4.5 micromoles produced during the first two hours. Boiled enzyme preparations were just as active as unboiled ones, but in both there was a definite stimulus over the spontaneous rate. Such an experiment does not rule out the production of some hydroxypyruvate, although a double step decarboxylation is indicated.

The second line of evidence of the slow formation of glycolaldehyde from DHF non-enzymatically can be detected by using crystalline alcohol dehydrogenase as a reagent to determine the glycolaldehyde concentration (9). If equal amounts of DHF and glycolaldehyde (0.2 micromoles) were added to crystalline alcohol dehydrogenase at pH 7.4, the DPNH is rapidly oxidized by the glycolaldehyde while there is a slow but steady oxidation of DPNH in the presence of added DHF. This rate amounts to about 0.035 micromoles of glycolaldehyde per hour under anaerobic conditions, and slightly less under aerobic conditions.

Chemical tests for phenylhydrazones or for glycolaldehyde (diphenylamine test, 14) were difficult to interpret for reasons already discussed, and because any DHF left in the preparation could give rise to glycolaldehyde during the actual analytic test itself. After vigorous aeration of the enzymatic mixture to autoxidize any remaining DHF, about 20 % of the original DHF was analyzed as glycolaldehyde.

Unsuccessful attempts were made to identify tartrate as the reaction product when DHF and DPNH were incubated with plant enzymes. When coupled with alcohol oxidation at pH 7.4 to generate the DPNH, considerable coupling occurred as indicated by the amount of acetaldehyde formed, but it was not possible to identify the product chromatographically as a tartrate (pH spray) or by the sensitive sodium vanadate test (34). One might expect that the product formed would be the meso-isomer because this is more active in reducing DPN. The vanadate test will analyze only the (−)-, (+)-, and (±)- forms of tartrate, and negative results indicate that little (−)-tartrate was accumulating.

Side reactions could also remove the DHF or the tartrate itself. These might include the decarboxylation of DHF to glycolaldehyde with the subsequent reduction to ethylene glycol via DPNH, or the tartrate could be converted to oxalacetate by dehydration (17, 20) followed by reduction to malate in the presence of DPNH and malic dehydrogenase.

Preliminary evidence that this latter reaction does occur in pea seed preparations has been obtained. When DPNH is incubated with (−)- or meso-tartrate, the DPNH is oxidized. The (+)- form is inactive. Further work is in progress to clarify this side reaction which may be similar to that reported in bacteria (17, 20). If this reaction converting tartrate to oxalacetate is further validated, the interest of tartaric dehydrogenase activity in plant metabolism is greatly enhanced.

Distribution of Tartaric Acid Dehydrogenase: Tartaric acid dehydrogenase activity has been demonstrated in pea plants, wheat embryos, and bean leaves. In peas, the activity is distributed throughout the plant, being found in mature roots and shoots, as well as in seeds soaked in distilled water for 12 hours. The pattern of distribution, therefore, appears to be similar to that of malic and hydroxymalonic acid dehydrogenase activities.

The intracellular distribution of tartaric acid dehydrogenase is similar to that of malic dehydrogenase, but differs from that for hydroxymalonic acid dehydrogenase. About 20 to 30 % of the original activity with meso-tartrate was associated with a particulate fraction, the remaining portion being found in the final supernatant solution. The initial separations were made after grinding the peas for 1 minute in a Waring blendor in an iced mixture of 0.5 M sucrose in 0.025 M phosphate buffer (pH 7.4). The particulate fraction was a washed sediment of particles centrifuged down between 500 to 25,000 × g for 30 minutes. In order to lower blank reactions and to demonstrate a DPN-dependency in the case of the particulate fraction, it was necessary to purify all fractions by ammonium sulfate precipitation. The fraction used for assay was that precipitated between 200 to 400 grams of ammonium sulfate per liter of enzyme solution, and then resuspended in 0.025 M phosphate buffer at pH 7.4. The activities were followed spectrophotometrically at pH 9.0 in the presence of 0.4 mg DPN, 200 micromoles of substrate (plus 5 micromoles of KCN in the case of added malate). Approximately 85 % of the total protein of the homogenate was recovered in the two fractions.

Although the ratio of activities with added tartrate and with hydroxymalonate were close to 1.0 in the supernatant fractions, no activity could be detected with added hydroxymalonate in the particulate fraction. The ratio of the activity with malate to that with tartrate varies from about 800 to 1300 in the different fractions.

In the other plant enzyme preparations used in

this study, the ratios of activity with hydroxymalonate to that with tartrate were always close to 1 when both activities were detectable. When the ratios of the activity with malate to that with tartrate were compared, the values ranged from 200 to 2500. Although this might indicate the presence of separate enzymes, changes in the side reactions could account for such variation. The question of the number of enzymes involved, therefore, is still open.

COMPETITIVE INHIBITION OF MALIC DEHYDROGENASE ACTIVITY: In experiments performed as described for hydroxymalonate (32), meso-tartrate was found to be a competitive inhibitor of malic dehydrogenase activity in pea seed preparations with a K_I value of approximately 2×10^{-2} M. (K_I equals the dissociation constant of the enzyme-inhibitor complex.) The values of K_I reported for inhibition by hydroxymalonate was about 1×10^{-2} M; the difference between these values is not considered highly significant. If present in high enough concentration in comparison with malate, therefore, both hydroxymalonate and tartrate might function as physiological regulators of malic dehydrogenase activity. (The value of K_m for L-malate, or the dissociation constant of the enzyme - substrate complex, was reported as 0.5×10^{-2} M (32)).

SIGNIFICANCE OF DHF IN PLANT METABOLISM: Some of the numerous enzymatic and non-enzymatic reactions of DHF are summarized in figure 3. DHF is shown here as a compound capable of being oxidized, reduced, or decarboxylated, with further similar reactions involving most of the initial products. At least 7 different dehydrogenase activities in plants are associated with these compounds. Some of these breakdown products, like glycolaldehyde and hydroxypyruvate, have been postulated as possible precursors of carbohydrates (10, 12, 25), and DHF oxidase (1, 24) plus diketosuccinic acid reductase can serve as a potential oxidase system comparable to the complex

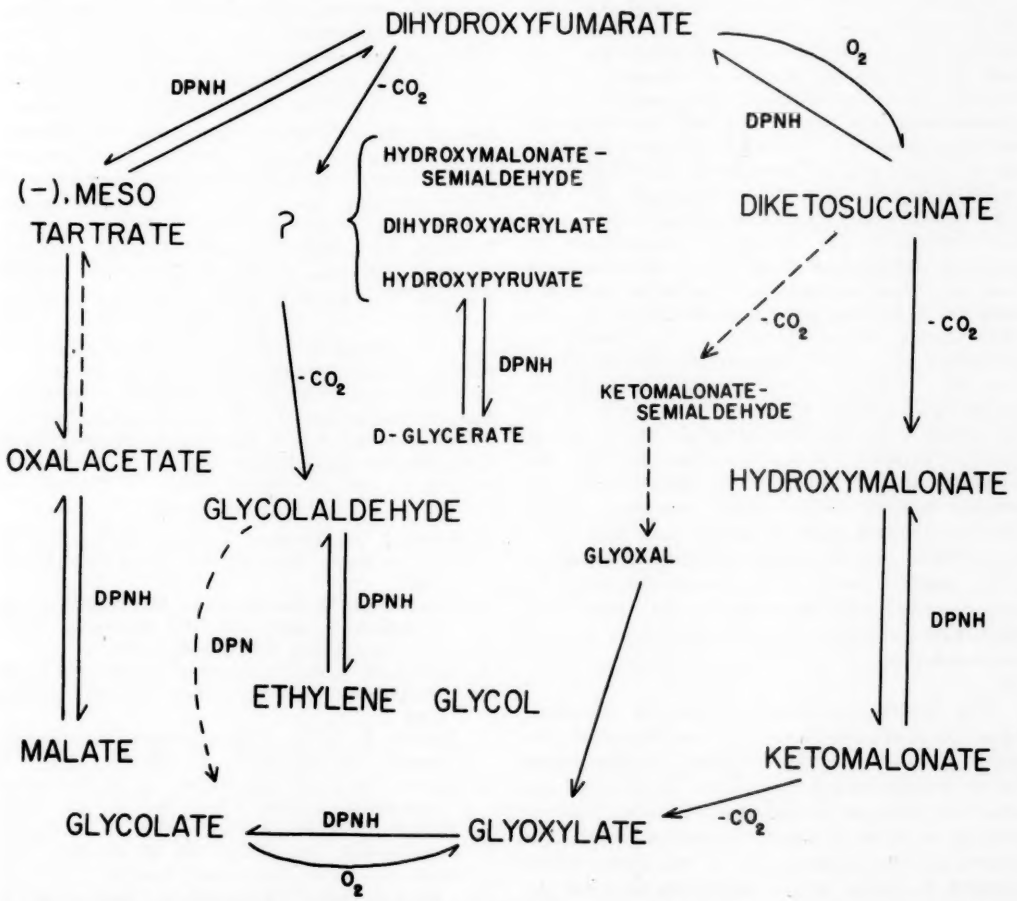

FIG. 3. Possible reactions of dihydroxyfumarate. Solid lines represent enzymatic or non-enzymatic activities reported in higher plants; dotted lines represent activities reported in animal tissues or which are postulated as possible chemical reactions.

involving glycolate and glyoxylate (36). Furthermore, transaminase reactions can connect some of these organic acids with their corresponding amino acids (26). DHF can be considered, therefore, as a possible key metabolite in plants.

The aerobic oxidation of DHF to diketosuccinate can occur non-enzymatically or enzymatically (1, 11, 18, 30). Whether the latter reaction is catalyzed by a separate oxidase or only by a peroxidase acting as an oxidase, and whether a metal ion is necessary for all such oxidations has not yet been definitely proven (24). The product of these oxidations is presumably diketosuccinate, but again this has never been unequivocably demonstrated. If any H_2O_2 is produced, the situation is further complicated by the subsequent oxidation by H_2O_2 of compounds such as hydroxymalonate to ketomalonate. The dehydrogenase reaction which reduces DHF to tartrate and the tentative evidence of the conversion of tartrate to oxalacetate in plants have already been discussed.

The direct decarboxylation products of DHF and diketosuccinate need further study, especially in relation to metal ion effects and to pH differences. The early chemical work of Fenton (4) identified glycolaldehyde and hydroxymalonate as the main decarboxylation products of the acids of DHF and diketosuccinate, respectively. Although preparations from higher plants catalyze non-enzymatically these decarboxylations at neutral pH, no enzymatically catalyzed reactions have been demonstrated, except in the more complicated Mn^{++}-phenol-peroxidase system reported by Kenten and Mann (11). Yeast preparations, on the other hand, are reported to decarboxylate DHF forming glycolaldehyde and CO_2 (23), while yeast pyruvic carboxylase can apparently act on hydroxypyruvate by converting it to glycolaldehyde (9). This latter reaction would make it difficult to determine whether hydroxypyruvate were the intermediate in the decarboxylation of DHF to glycolaldehyde in some plant preparations, although the ones used in this study had no pyruvic acid carboxylase activity without added coenzyme. While Fenton considered either hydroxypyruvic acid, dihydroxyacrylic acid or hydroxymalonate-semialdehyde as the intermediate in this double decarboxylation, he gives no evidence for their detection (6). Kun's data identifying glyoxylate and hydroxypyruvate as non-enzymatic products have already been discussed (15, 16).

The spontaneous decarboxylation of diketosuccinate to hydroxymalonate has been frequently reported (5, 6, 16), and an enzymatic decarboxylation can be demonstrated in yeast (22). Fenton cites evidence for ketomalonate-semialdehyde as an intermediate in a metal catalyzed conversion of DHF to glyoxal via diketosuccinate (5). If any such reaction occurred in plants, glyoxal could then be converted to glyoxylate (13). Another possible route of glyoxylate production is by a DPN-linked aldehyde oxidase reaction which oxidizes glycoaldehyde to glycolic acid as reported in liver (14). No such activity could be demonstrated spectrophotometrically using the plant preparations described in this study.

The author feels that any further enzymatic work with DHF and its derivatives must be preceded by a thorough chemical study of all these compounds.

Summary

A dehydrogenase activity which oxidizes (−)- or meso-tartrate to either the enol or keto form of dihydroxyfumarate has been demonstrated in pea, bean and wheat extracts. This activity has been measured spectrophotometrically with substrate amounts of DPN^+ or colorimetrically by the Prussian blue analysis of the $K_4Fe(CN)_6$ formed in a system coupled with the oxidation of $K_3Fe(CN)_6$. Under the conditions used, neither Mg^{++} nor ethylenediamine-tetraacetic acid affects the rate of this activity. The reduction of dihydroxyfumarate to tartrate in the presence of DPNH can be demonstrated spectrophotometrically under anaerobic conditions in certain preparations. Identification of the products of the dehydrogenase activity is hampered by at least three side reactions: the decarboxylation of dihydroxyfumarate to glycolaldehyde; the autoxidation to diketosuccinate followed by decarboxylation to hydroxymalonate; the possible dehydration of tartrate to oxalacetate. No evidence of significant amounts of glyoxylate or hydroxypyruvate was obtained. The dehydrogenase activity is found in seeds, roots, and shoots of peas. Although the activity is associated mainly with the supernatant fraction, a definite activity can be demonstrated in a partially purified particulate fraction sedimented between 500 to 25,000 × g. The possibility that this activity is merely a secondary one of the similarly distributed malic dehydrogenase has not yet been eliminated. Like hydroxymalonate, tartrate is a competitive inhibitor of malic dehydrogenase activity. The possible key role of dihydroxyfumarate in plant metabolism is discussed.

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THE INFLUENCE OF SALTS ON PYRUVATE KINASE FROM TISSUES OF HIGHER PLANTS[1,2]

GENE MILLER[3] AND HAROLD J. EVANS

BOTANY DEPARTMENT, NORTH CAROLINA STATE COLLEGE, RALEIGH, NORTH CAROLINA

The enzyme pyruvate kinase which catalyzes the reversible transfer of phosphate from phosphoenolpyruvate (PEPA) to adenosine diphosphate (ADP) yielding pyruvate and adenosine triphosphate (ATP) is widely distributed in living organisms (2, 3, 4, 8, 9, 11, 15). Lohmann and Meyerhof (11) in 1934 demonstrated that the activity of pyruvate kinase from rat muscle was dependent on the presence of Mg^{++} in the assay medium. Utter and Werkman (22) reported that extracts from *Escherichia coli* contained pyruvate kinase and that it was activated by Mg^{++} or Mn^{++}. In their experiments Ni^{++}, Na^{+} and K^{+} were either without effect or were inhibitory.

The K^{+} activation of pyruvate kinase from rat muscle was shown initially by Boyer, Lardy and Phillips (3, 4) and later by Lardy and Ziegler (9). The complete dependence of the activity of the enzyme on univalent cations was demonstrated first by the work of Kachmar and Boyer (8). This requirement was in addition to that for divalent cations. K^{+}, NH_4^{+} or Rb^{+} satisfied the univalent cation requirement but Na^{+}, Li^{+} or Ca^{++} counteracted the activation. In the absence of K^{+}, Na^{+} additions resulted in a small but significant stimulation. Boyer (2) reported that extracts from eleven different vertebrate and invertebrate organisms contained pyruvate kinase and that the enzyme from all sources was strongly activated by K^{+}. The optimum concentrations of univalent cations in molarity were 0.15, 0.01 and 0.05 for activity of the enzyme from muscles of rabbit, Anodonta and Limulus, respectively.

Introductory studies (14) on the fermentation of phosphoglyceric acid by dialyzed yeast extracts showed no univalent cation activation. Later, Seitz (18) reported that yeast extracts that had been thoroughly dialyzed failed to ferment phosphoglyceric acid unless K^{+} or NH_4^{+} was supplied. In the absence of univalent cations the phosphoglyceric acid fermentation stopped at the phosphoenolypyruvate stage. With this system NH_4^{+} was more effective than K^{+} as an activator which is in contrast to observations with the enzyme from other sources.

Stumpf (20) and Tewfik and Stumpf (21) have provided indirect evidence for the presence of pyruvate kinase in higher plants. Extracts of an acetone powder of pea seed or leaves contained enzymes that catalyzed the conversion of phosphoglyceric acid to pyruvate. When dialyzed extracts were used, the addition of adenosine monophosphate (AMP) increased the rate of formation of pyruvate from phosphoglyceric acid. The effects of ADP or univalent cation additions to the system were not indicated in these studies.

It is apparent that pyruvate kinase is widely distributed in microorganisms and tissues of animals and indirect evidence suggests that this enzyme is present in higher plants. In extracts of animal tissues and yeast the activation of the enzyme by K^{+} and other univalent cations seems to be a general phenomenon representing a fundamental property of the enzyme. The experiments that have been conducted with higher plants have not been specifically designed to demonstrate cation requirements of the system. It was deemed necessary therefore, to conduct a systematic study of the influence of various salts on pyruvate kinase obtained from several higher plant species. In view of the limited available information on the role of univalent cations in the metabolism of higher plants, this study was considered especially appropriate.

MATERIALS AND METHODS

PREPARATION OF EXTRACTS: The enzyme extract used in most of the experiments reported herein was prepared from an acetone powder of Alaska pea seed (20). The seed were soaked in distilled water at room temperature for 5 hours then removed from the water and homogenized for 3 minutes in acetone at $-10°$ C by use of an Omni mixer (Ivan Sorvall Inc., Norwalk, Conn.). The acetone powder of this material was prepared as previously described (5). For the preparation of the extract, 4 gm of acetone powder were homogenized in 15 ml of 0.05 M tris (hydroxymethyl) aminomethane–hydrochloride buffer (TRIS) at pH 7.4 and the mixture centrifuged at $25{,}000 \times g$ for 10 minutes. The supernatant was dialyzed for 4 hours against 4 liters of 0.01 M TRIS buffer at pH 7.4, and used as a source of the enzyme. When extracts from pea seed were dialyzed for longer periods the activity was lost. The addition of glutathione or versene or both to the dialysis solution had no effect on enzyme stability. Since the enzyme activity of extracts declined rapidly, daily preparations were necessary. The extracts prepared by the described procedure contained 12 to 14 mg proteins per ml as determined by Folin's phenol reagent (12).

Extracts from various tissues of other species were prepared for the experiments related to the occurrence of pyruvate kinase in higher plants. Five grams of the fresh tissue were homogenized in 15 ml of 0.05 M TRIS pH 7.4 and strained through cheesecloth. The extract was centrifuged for 10 minutes at $25{,}000 \times g$. The lability of the enzyme from the various plant species differed and accordingly the lengths of the

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[3] Present address: Department of Botany and Plant Pathology, Utah State Agricultural College, Logan, Utah.

dialysis periods varied with the different preparations (table II).

Since pyruvate kinase activity was low in extracts from certain plant materials, concentration of the enzyme was necessary. This was accomplished by precipitation of protein by the addition of 50 ml of acetone at – 15° C for each 20 ml of crude extract. The precipitate was collected by filtration rewashed with cold acetone and allowed to dry at room temperature. The dried precipitate from each 20 ml aliquot of original extract was taken up in 5 ml of 0.05 M TRIS buffer pH 7.4 and centrifuged at 25,000 × g for 10 minutes. The supernatant was used as a source of enzyme and is referred to as the acetone precipitated extract.

Other Materials: The TRIS salt of phosphoenolpyruvate acid (PEPA) was prepared from the silver-barium double salt (California Foundation for Biochemical Research of Los Angeles) by trituration in an ice bath with a 5 to 10 % excess, on an equivalent basis, of *N* HCl (8). This was followed by the addition of a slight excess of M TRIS sulphate. The precipitate was removed by centrifugation and the clear solution was adjusted to pH 7.4 using TRIS buffer, then diluted to a concentration of 0.015 M. In later experiments the cyclohexylamine salt of PEPA was used and the results were identical with those obtained with TRIS PEPA.

The barium salts of ADP and AMP were purchased from the Sigma Chemical Company of St. Louis. TRIS ADP was prepared by dissolving the barium salt in 0.1 M HCl and precipitating Ba^{++} by addition of a calculated amount of TRIS sulphate. The solutions of TRIS ADP and TRIS AMP were adjusted to pH 7.4 using TRIS buffer and diluted to a concentration of 0.05 M. Buffer solutions were prepared as previously described (13).

Inorganic Analyses: Aliquots of the dialyzed extract from the acetone powder of pea seed were ashed in a muffle furnace at 600° C and analyzed for Na and K by use of a Perkin-Elmer flame photometer (24). NH_3 was determined after distillation from alkaline extracts by Nesselerization (7).

Standard Assay Procedure: Enzyme activity was assayed by determination of the pyruvate formed from PEPA after incubation of the various reactants with the enzyme. In this procedure ADP was added directly to reaction mixtures instead of addition of the hexokinase system for the generation of ADP from ATP as reported by other workers (8). The omission of hexokinase was necessary since the work of Hers (6) and unpublished experiments by the authors have shown that hexokinase activity is influenced by both univalent and divalent cations. Evidence for the validity of the standard procedure is presented under Results.

The reaction mixture in a final volume of 1 ml contained the following constituents in micromoles: 50 of TRIS buffer at pH 7.4; 1.5 of TRIS or cyclohexylamine salt of PEPA; 2.5 of TRIS ADP; 8.0 of $MgSO_4$; 50 of KCl and enzyme extract usually containing 0.6 to 0.7 mg protein. The mixtures were incubated at 37° C for 10 minutes and the reaction stopped by the addition of 1 ml of cold 0.0125 % 2,4-dinitrophenyl hydrazine (8). Pyruvate was estimated by the method described by Kachmar and Boyer (8). Precipitates of protein or $Mg(OH)_2$ in the reaction mixtures were removed by centrifugation in order to prevent interference with the colorimetric determination. In each assay the pyruvate formed under the desired experimental conditions was compared with a control reaction containing all reactants except ADP. This served to correct for pyruvate formation resulting from the action of phosphatases. No pyruvate was formed when the enzyme extracts were boiled and added to reaction mixtures with or without the various experimental additions. The various experiments were replicated at least three times and the results averaged. The error between determinations was less than 10 %.

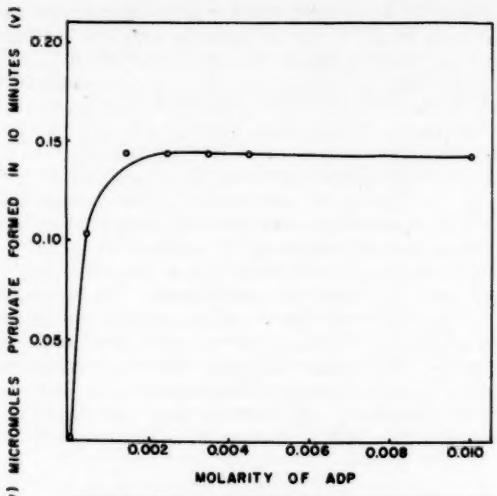
Results

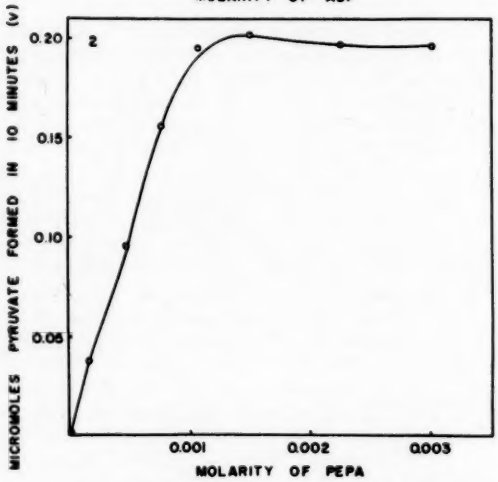
Validity of the Standard Assay Procedure: Since the literature contains no detailed studies of the properties of pyruvate kinase from higher plants it was essential to determine the concentrations of ADP and PEPA necessary for saturation of the enzyme. As shown in figure 1, a concentration of 1.4×10^{-3} M ADP was required for maximum activity. The K_M for ADP estimated from the saturation curve is 5.0×10^{-4}. When AMP was used instead of ADP with dialyzed enzyme from pea seed no activity was observed. With the undialyzed enzyme extract, however, the addition of AMP resulted in weak activity.

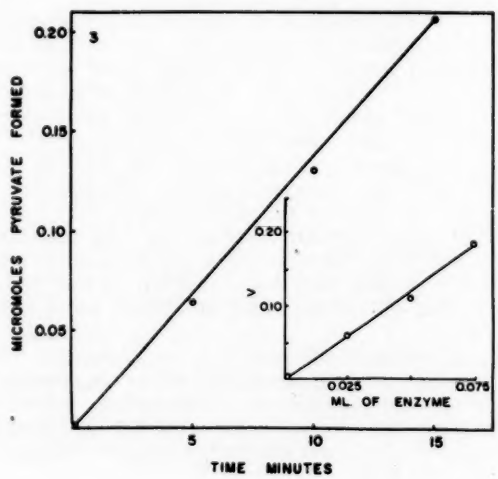
The influence of various concentrations of PEPA on the enzymatic rate of pyruvate formation is shown in figure 2. The final concentration of PEPA required for the maximum rate was 1.3×10^{-3} M which is in good agreement with the figure reported for pyruvate kinase from rabbit muscle (8). The K_M for PEPA estimated from the curve in figure 2 is 3×10^{-4} M.

Under conditions of the standard assay the rate of pyruvate formation remained linear for at least the initial 15 minutes as shown in the major graph of figure 3. The inset in figure 3 provides evidence that the initial velocity (v) of the reaction was proportional to enzyme concentration. At the end of 15 minutes (major graph of figure 3) only 13 % of the substrate had undergone reaction. Kachmar and Boyer (8) reported the reaction velocity was linear with enzyme concentration and with time when less than 40 % of the total PEPA present had reacted. In the various experiments reported in this study less than 40 % of the PEPA was utilized during each assay.

The effect of pH on the activity of pyruvate kinase from pea seed was studied within the range 6.0 to 9.0 using phosphate and TRIS buffers. A broad optimum was observed between pH 7.0 and 9.0 and therefore pH 7.4 was arbitrarily chosen for the standard procedure.







Evidence that the concentrations of K^+ and Mg^{++} used in the standard assay procedure were sufficient for maximum activation is presented in the following sections of this paper. Since the results to be presented indicate that cations such as K^+ and Mg^{++} possess activating capacities for the enzyme and that anions such as Cl^- and $SO_4^=$ have no specific effect, the symbols for the cations are used in describing influences of cation salts on the system.

UNIVALENT CATION ACTIVATION OF THE ENZYME FROM PEA SEED: The curves in figure 4 A illustrate the influence of univalent cations added as the chloride salts on enzyme activity. In figure 4 B the data are plotted by the method of Lineweaver and Burk (10) and from these curves the Michaelis constants (K_A) for the activating univalent cations were calculated. When the data were plotted in figure 4 B only those points on the curves in figure 4 A that were free of apparent inhibition were utilized. In the absence of added salts, enzyme activity was approximately 10 % of the maximum attainable when an optimum concentration of KCl was added. Since the enzyme was labile, prolonged dialysis was not possible and analyses indicated that the dialyzed extract from pea seed contained 8×10^{-4} M K^+, no detectable NH_4^+ and 2.2×10^{-3} M Na^+. This was sufficient to account for the enzyme activity observed in the absence of added salts. Maximum enzyme activity with the chloride salts of either K^+, Rb^+ or Na^+ was exhibited at a concentration of 0.05 M, and greater concentrations reduced the activity. K_A values for K^+, Rb^+ and Na^+ were 2.4×10^{-3}, 4.2×10^{-3} and 1.4×10^{-3} M, respectively. Maximum activation with NH_4^+ was observed at a concentration of 0.035 M and greater concentrations caused a decline in activity. The K_A for NH_4^+ was calculated to be 1.2×10^{-3} M. The addition of Na^+ to reaction mixtures resulted in considerable stimulation of pyruvate kinase activity, but the

FIG. 1. The effect of ADP concentration on the rate of the enzyme reaction. The standard assay procedure was used with variation in concentration of ADP as indicated. The dialyzed enzyme extract from pea seed added to each reaction mixture contained 0.6 mg protein.

FIG. 2. The effect of concentration of PEPA on the rate of the enzyme reaction. The standard assay procedure was used with variation in concentration of PEPA as indicated. The dialyzed enzyme extract from pea seed added to each reaction mixture contained 0.6 mg protein.

FIG. 3. (*Major graph*) Proportionality of enzyme activity with time. The standard assay procedure was used with variation in the time as indicated. The enzyme extract from pea seed added to each reaction mixture contained 0.6 mg protein. (*Inset*) Proportionality of enzyme activity indicated as initial velocity (v), with enzyme concentration. The standard assay procedure was used with variation in quantity of enzyme extract added to each reaction mixture as indicated. The dialyzed extract from pea seed contained 13 mg protein per ml.

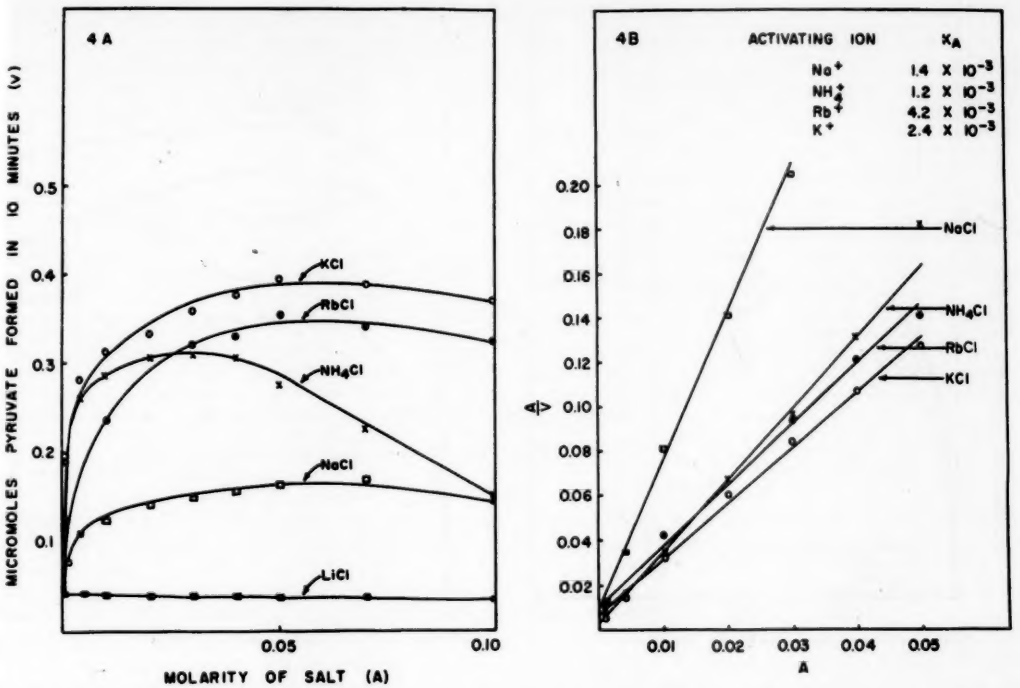


FIG. 4 A. The influence of various concentrations of univalent cation salts on the activity of pyruvate kinase. The standard assay procedure was used with variation in univalent cation salts as indicated. The dialyzed enzyme extract from pea seed added to each reaction mixture contained 0.6 mg protein.

FIG. 4 B. The data of figure 4 A plotted by the method of Lineweaver and Burk. Calculation of regression lines by the method of least squares for the various chloride salts gave the following regression equations: Na^+, $Y = 9.3 \times 10^{-3} + 6.6\,X$; NH_4^+, $Y = 3.3 \times 10^{-3} + 3.1\,X$; Rb^+, $Y = 11.3 \times 10^{-3} + 2.7\,X$; K^+, $Y = 6.3 \times 10^{-3} + 2.5\,X$.

magnitude of the effect was much less than that obtained with the chloride salts of K^+, Rb^+ or NH_4^+. Li^+ was completely ineffective as an activator for the system.

It has been reported that quantities of NH_4^+ greater than 0.5 micromole per ml interfere with the pyruvate kinase assay method (8) that included the hexokinase system. In the assay procedure reported here concentrations of NH_4^+ ranging from 1 to 100 micromoles per ml had no effect on the standard assay system. Unpublished experiments by the authors have demonstrated that NH_4^+ interferes with hexokinase assays determined by glucose disappearance. The hexokinase system was not included in the pyruvate kinase assay system used in these studies and perhaps this may account for the lack of interference under our conditions.

EFFECT OF VARIOUS ANIONS ON ACTIVITY OF ENZYME FROM PEA SEED: Univalent cation salts differed in their influence on pyruvate kinase activity, and the effect was primarily independent of the anion present as shown in table I. The Cl^-, NO_3^-, $SO_4^=$ and phosphate (mixture of $HPO_4^=$ and $H_2PO_4^-$) (13) salts of K^+ gave similar results throughout the experimental range of concentrations. I^- and to a lesser extent the Br^- salts became inhibitory before the maximum activation of 0.05 M K^+ was reached.

ACTIVATION OF ENZYME FROM PEA SEED BY DIVALENT CATIONS: The effect of various divalent cation

TABLE I

THE EFFECT OF VARIOUS CONCENTRATIONS OF DIFFERENT POTASSIUM SALTS ON THE ACTIVITY OF PYRUVATE KINASE FROM PEA SEED *

SALT	CONC OF ADDED SALT (N)						
	0.00	0.001	0.003	0.01	0.03	0.05	0.10
	Micromoles pyruvate formed in 10 min						
KCl	0.015	0.082	0.120	0.160	0.192	0.228	0.220
KNO_3	0.020	0.078	0.117	0.156	0.195	0.234	0.221
KBr	0.018	0.065	0.111	0.142	0.189	0.196	0.189
KI	0.015	0.072	0.117	0.140	0.156	0.156	0.091
K_2SO_4	0.020	0.070	0.125	0.156	0.190	0.221	0.214
K_2HPO_4–KH_2PO_4	0.016	0.069	0.120	0.160	0.200	0.236	0.225

* The standard assay procedure was used with variation of potassium salt as indicated. The dialyzed pea seed extract added to each reaction mixture contained 0.6 mg protein.

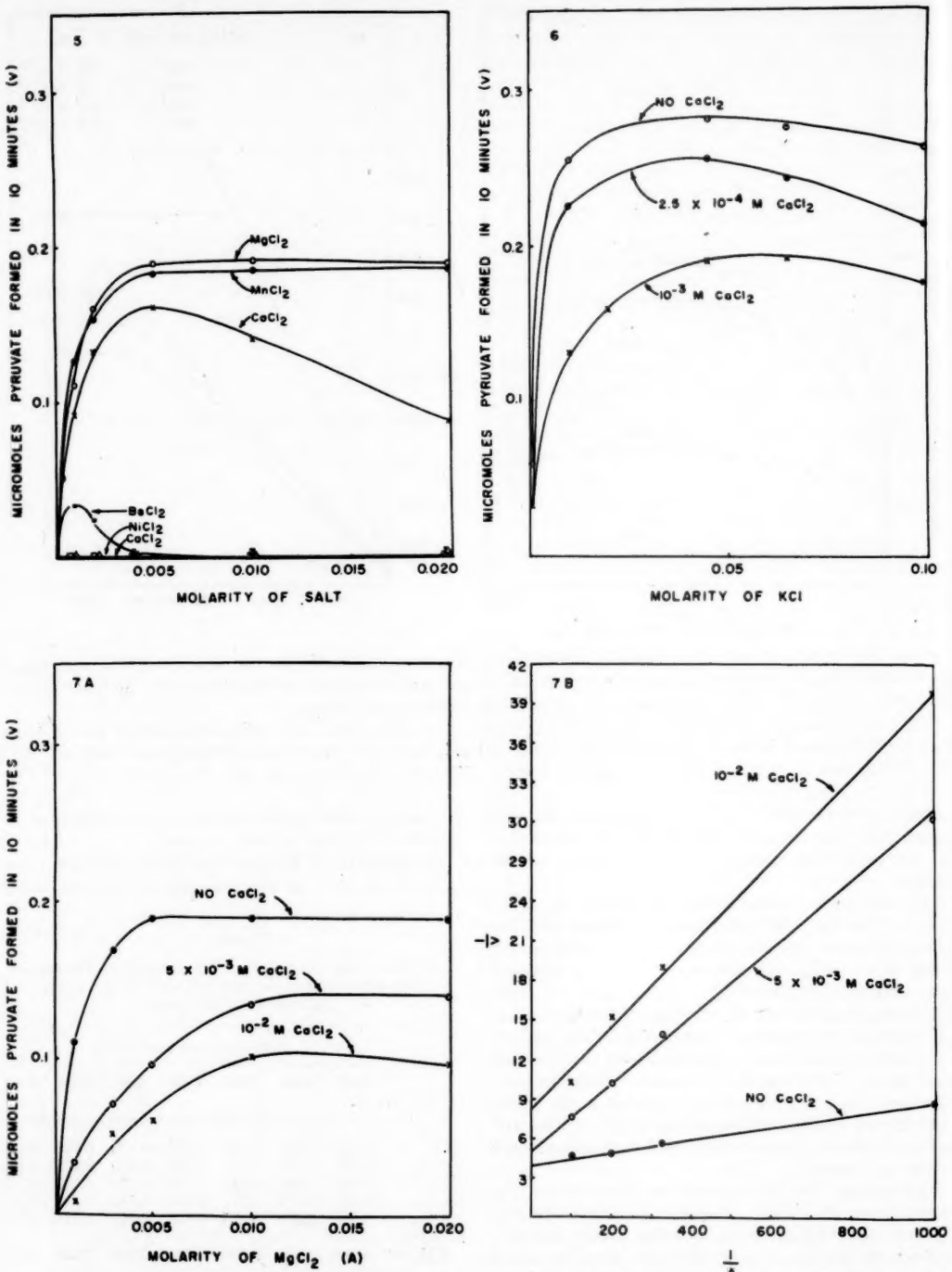


FIG. 5. The activation of pyruvate kinase by various concentrations of divalent cation salts. The standard assay procedure was used with variation in concentration of divalent cation salts as indicated. The dialyzed enzyme extract from pea seed added to each reaction mixture contained 0.6 mg protein.

FIG. 6. The inhibition of pyruvate kinase by $CaCl_2$ at various concentrations of KCl. The standard assay pro-

TABLE II

ACTIVATION OF PYRUVATE KINASE FROM VARIOUS SOURCES BY POTASSIUM CHLORIDE

PLANT SPECIES	PORTION OF PLANT	KCl ADDED TO ASSAY MEDIUM (M)						
		0.00	0.001	0.004	0.01	0.03	0.05	0.10
		Micromoles pyruvate formed in 10 min/mg protein						
Triticum vulgare	Germ * (embryo)	0.026	0.156	0.208	0.416	0.450	0.480	0.416
Nicotiana Tabacum	Leaf **	0.026	0.038	0.052	0.078	0.104	0.116	0.052
Gossypium barbadense	Seed †	0.0	0.030	0.060	0.213	0.195	0.195	0.172
Avena sativa	Seed **	0.130	0.260	0.380	0.520	0.600	0.726	0.520
Avena sativa	Seed *	0.0	0.117	0.195	0.216	0.273	0.300	0.216
Zea Mays	Seed *	0.0	0.117	0.136	0.177	0.215	0.205	0.177
Pisum sativum	Seed **	0.081	0.172	0.227	0.256	0.307	0.358	0.305
Pisum sativum	Leaf *	0.110	0.120	....	0.222	0.289	0.336	0.286
Apium graveolens	Petiole ††	0.026	0.035	0.045	0.078	0.120	0.200	0.182
Beta vulgaris	Seed ††	0.035	0.090	0.120	0.175	0.260	0.310	0.310

* Crude extract dialyzed 4 hrs against 0.01 M TRIS buffer, pH 7.4.
** Acetone precipitate of the crude extract.
† Crude extract dialyzed 24 hrs against 0.01 M TRIS buffer, pH 7.4.
†† Crude undialyzed extract.

chlorides on pyruvate kinase activity is illustrated by the curves in figure 5. In these experiments the standard assay procedure was used with the exception of the variation in divalent cation salt as indicated (fig 3). The activating capacities of Mg^{++} and Mn^{++} were approximately equal at all concentrations tested and the maximum enzyme activity was observed at a concentration of 5×10^{-3} M. The K_A calculated for either Mg^{++} or Mn^{++} was 9×10^{-4} M. Co^{++} was an effective divalent cation activator at concentration up to 5×10^{-3} M. This concentration was optimum and resulted in approximately 87 % of the activity obtained with equal concentrations of either Mg^{++} or Mn^{++}. Concentrations of Co^{++} greater than 5×10^{-3} M caused a sharp decline in activity. A slight activation with Be^{++} was observed at concentrations near 10^{-3} M, but concentrations greater than 4×10^{-3} M were completely ineffective. Ni^{++} and Ca^{++} salts exhibited no activating capacity at concentrations ranging up to 0.2 M.

INTERACTION OF CATIONS ON THE ENZYME FROM PEA SEED: The inhibitory action of Ca^{++} at various concentrations of K^+ is illustrated by the curves in figure 6. When the reaction mixtures contained K^+ at a concentration of 0.01 M, a concentration of 10^{-3} M Ca^{++} inhibited the activity approximately 50 %. An increase in the K^+ concentration up to 0.07 M resulted in a decline in the inhibition to 30 %. Greater concentrations of K^+ resulted in no further reversal of the Ca^{++} inhibition. With K^+ at 0.01 M and Ca^{++} at 2.5×10^{-4} M in the assay medium approximately 13 % inhibition of pyruvate kinase was manifested. Greater concentrations of K^+ did not reverse this inhibition to any appreciable extent. The data in figure 6 were plotted by the reciprocal method (10) and it was concluded that the inhibition by Ca^{++} was primarily but not completely, noncompetitive with respect to K^+.

Ca^{++} inhibition of pyruvate kinase at various Mg^{++} concentrations is illustrated by the curves in figure 7 A. When the Mg^{++} concentration was 10^{-3} M and Ca^{++} added at concentrations of 0.01 and 5×10^{-3} M, inhibitions of 92 and 70 %, respectively were obtained. Increasing the Mg^{++} concentration of 0.01 M partially reversed the inhibition, resulting in 46 and 28 % inhibition from Ca^{++} concentrations of 10^{-3} M and 5×10^{-3} M, respectively. Further reversal of the inhibition by Ca^{++} at these two concentrations was not possible by increasing the Mg^{++} concentration in assays. The curves plotted by the Lineweaver and Burk method (10) (fig 7 B) and obtained from data in figure 7 A, suggests that Ca^{++} inhibition of pyruvate kinase is complex and probably consists of a combination of both competitive and non-competitive types of inhibition.

Pyruvate kinase activity of pea seed acetone powder determined with the concentrations of K^+ and Mg^{++} used in the standard assay procedure was not influenced by the addition of Li^+ or Na^+ at concentrations up to 0.2 M. These results are not in accord with those reported for the enzyme from rabbit muscle (8).

K^+ ACTIVATION OF ENZYME FROM OTHER SPECIES: Pyruvate kinase activity was found to be present in extracts of a variety of plant species. In general seeds were the best sources of the enzyme, but consid-

cedure was used with variation in concentrations of KCl and $CaCl_2$ as indicated. The dialyzed enzyme extract from pea seed added to each reaction mixture contained 0.6 mg protein.

FIG. 7 A. The inhibition of pyruvate kinase by $CaCl_2$ at various concentrations of $MgCl_2$. The standard assay procedure was used with variation in concentration of $MgCl_2$ and as indicated. The dialyzed enzyme extract from pea seed added to each reaction mixture contained 0.6 mg protein.

FIG. 7 B. The data of figure 7 A plotted by the method of Lineweaver and Burk.

erable activity was found in leaves and other tissues as indicated in table II. Little or no activity was found in extracts of roots from the various plants. An absolute dependence of K^+ for activity was demonstrated for dialyzed extracts of cotton, oat and corn seeds. This evidence strongly supports the conclusion that K^+ or some other univalent cation serves as indispensable activator for pyruvate kinase of higher plants. It is apparent from the data of table II that the addition of KCl resulted in a striking increase in the activity of the enzyme from all the sources studied. All enzyme extracts except those from cotton seed were maximally activated with a K^+ concentration of 0.05 M. With the extract from cotton seed, however, 0.01 M K^+ was sufficient for the maximum rate. This lower cation requirement was observed consistently in several experiments. It was considered desirable, therefore, to carry out further studies with the enzyme from this source.

An extract was prepared from cotton seed and dialyzed for 20 hours as described under Materials and Methods. In contrast to extracts from other sources the activity of these preparations remained active after this relatively long dialysis period. The influence of various concentrations of the chlorides of K^+, Rb^+, NH_4^+ and Na^+ on the enzyme activity is presented graphically in figure 8 A. The data plotted by the Lineweaver and Burk method (10) is presented in figure 8 B. Maximum activation was observed with K^+ at 0.01 M and increased concentrations resulted in a slight decrease in activity. A concentration of 0.03 M NH_4Cl resulted in maximum activity with this salt and greater concentrations were less effective. The maximum activity of the enzyme was obtained with both Rb^+ and Na^+ at a concentration of 0.05 M, however, as observed with extracts from peas, the effectiveness of Na^+ as an activator was much less than that of K^+, NH_4^+ or Rb^+. The K_A values for Rb^+, NH_4^+ and Na^+ were of the same order of magnitude as those calculated for pea extracts, but the K_A value for K^+ was 1.5×10^{-3} M as compared to 2.4×10^{-3} M for the pea seed extract. Low concentrations of Rb^+ possessed less activating capacity than comparable concentrations of NH_4^+ or K^+, but the activating capacity of this cation approached that of K^+ and was greater than that of NH_4^+ at higher concentrations.

Discussion

The results of these experiments provide direct evidence that pyruvate kinase is widely distributed in the tissues of higher plants. The detailed studies of dialyzed extracts of pea seed indicate that pyruvate kinase from this material possesses properties

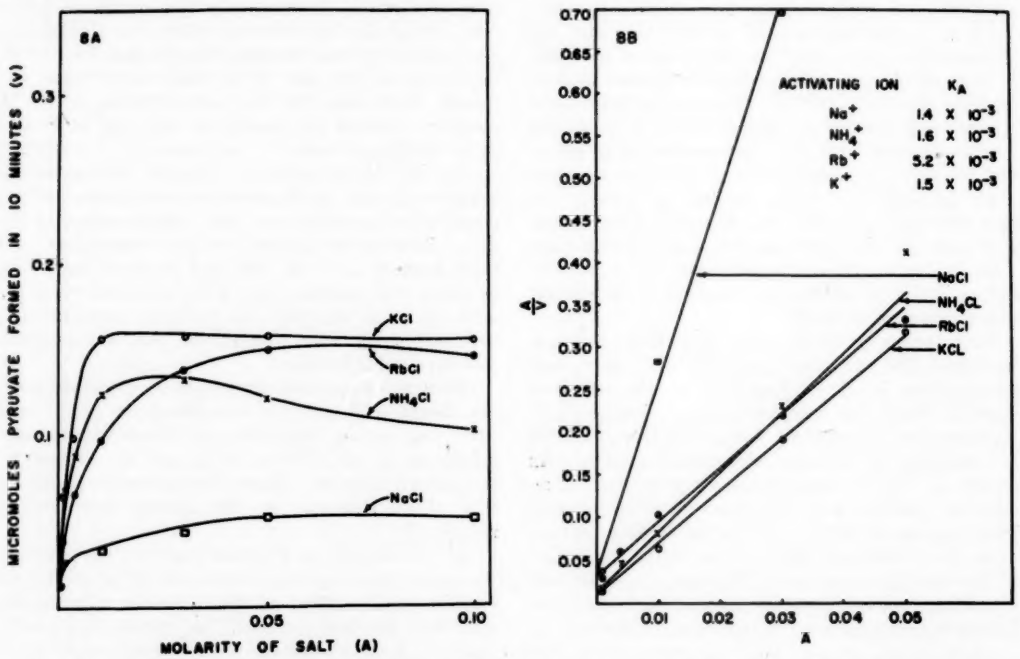


FIG. 8 A. The influence of various concentrations of univalent cation salts on the activity of pyruvate kinase from cottonseed extract. The standard assay procedure was used with variations in univalent cation salts as indicated. The enzyme extract (dialyzed for 20 hours) added to each reaction mixture contained 0.80 mg protein.

FIG. 8 B. The data of figure 8 A plotted by the method of Lineweaver and Burk. Calculation of regression lines by the method of least squares for the various chloride salts gave the following regression equations: Na^+, $Y = 0.032 + 22.5\,X$; NH_4^+, $Y = 0.011 + 7.1\,X$; Rb^+, $Y = 0.032 + 6.1\,X$; K^+, $Y = 0.009 + 6.1\,X$.

that are very similar to those reported for the enzyme from animals and yeast. An absolute requirement for divalent cations was established and the maximum activity was obtained with either Mg^{++} or Mn^{++} salts at a concentration of approximately 4×10^{-3} M. These findings correspond closely to those reported for the enzyme from *Escherichia coli* (22) and animal tissues (11). Co^{++} and to a small extent Be^{++} served as divalent cation activators. As far as the writers are aware the effects of these two cations on the enzyme from other sources has not been determined.

The system from all the plant sources investigated exhibited a striking requirement for univalent cations. The activity of the enzyme was essentially independent of the anion present with the exception of I^- and Br^-, which were slightly inhibitory at concentrations greater than 0.05 *N*. With the extracts from either pea or cotton seed, K^+, NH_4^+ or Rb^+ were effective activators and presumably NH_4^+ or Rb^+ would have substituted for K^+ as an activator for the enzyme from wheat, tobacco, oat, corn, celery and red beet which were the other species surveyed. The work of Kachmar and Boyer (8) on the rabbit muscle enzyme indicated that K_A values for K^+, NH_4^+ or Rb^+ were all near 0.011 M. Our calculations (fig 4 B) with the pea seed enzyme revealed K_A values of 2.4×10^{-3}, 1.2×10^{-3}, 4.2×10^{-3} and 1.4×10^{-3} M for K^+, NH_4^+, Rb^+ and Na^+, respectively, which are roughly one-fifth the value reported for the enzyme from rabbit muscle. The K_A values for these cations determined with the enzyme from cotton seed were of the same order of magnitude with exception of the value for K^+ which was 1.5×10^{-3} M. Even though the extracts from pea or cotton seed were not purified it seems apparent that the enzyme from these sources has a greater affinity for K^+, NH_4^+ or Rb^+ than does the enzyme from rabbit muscle. Boyer (2) has pointed out that the K_A for K^+ with the rabbit muscle enzyme is 37 times greater than that for the enzyme from the muscle of Anodonta. If one compares the velocities of the pea seed enzyme reactions at points where the maximum velocities were observed with the various cations relative values for: K^+, Rb^+, NH_4^+, Na^+, Li^+ are 100, 85, 76, 31, 0, respectively, and the comparable values for the cotton seed enzyme are almost the same. Certain of these relative rates may be compared with those obtained with rabbit muscle (8) which are: K^+, Rb^+, $NH_4^+ = 100$, 84, 72, respectively. In a comparison of the effects of univalent cations of the enzyme from pea seed (fig 4 A and B) with those of the enzyme from cotton seed (fig 8 A and B) it is apparent that the maximum velocity of the former was obtained at a concentration of 0.05 *N*, whereas the maximum velocity with the latter enzyme was observed at a KCl concentration of 0.01 *N*. A decision on the possible significance of this difference perhaps should be delayed until studies have been conducted with enzymes that have been highly purified.

Some of the observations concerning the capacities of various univalent cations to activate pyruvate kinase from plants may have a bearing on physiological problems that involve these elements. In the absence of other univalent cations (fig 4 A) Na^+ was a weak activator but Li^+ exhibited no activating capacity. In contrast to the results reported with pyruvate kinase from muscle (8) neither Na^+ nor Li^+ at concentrations up to 0.2 *N* counteracted the activating influence of K^+ at a concentration of 0.05 *N*. On the basis of these observations it would seem logical to conclude that pyruvate kinase in the animal organism may represent a locus of physiological antagonism between K^+ and Na^+. On the other hand, the stimulatory effects of low concentrations of these two cations on pyruvate kinase in plants would be expected to be additive. This point may be related to the well known capacity (23) of Na^+ salts to stimulate plant growth under conditions where the K^+ supply is limited. Since both Rb^+ and NH_4^+ are relatively efficient activators of pyruvate kinase and other enzymes such as phosphotransacetylase (19) and aldehyde dehydrogenase (1) it is necessary to consider the possible physiological role of these cations as activators for these enzymes in living plants. One would expect that either NH_4^+ or Rb^+ would substitute to some degree for K^+ as a univalent cation in plant metabolism. In this regard Richards (17) has reported that the addition of Rb^+ to cultures of barley decreased the K^+ requirements for growth. In considering the possible capacity of certain cations to substitute for others in specific enzymes of an organism, one must keep in mind the capacity of the organism, in general, to tolerate the concentrations of the particular cations that are required for activation of the specific systems.

The evidence presented in figures 6, 7 A and 7 B indicates that Ca^{++} is a relatively strong inhibitor of pyruvate kinase and that the inhibition is partially competitive with respect to both K^+ and Mg^+. This is interesting in view of the well known antagonistic effects of Ca^{++} toward both K^+ and Mg^{++} (16). The mechanism of the cation activation or inhibition of pyruvate kinase is not known, but from the work of Kachmar and Boyer (8) and Hers (6) one might speculate that Mg-ADP is the active cofactor and that Ca-ADP is inactive and competes to some extent for an active enzyme site. Ca^{++} also may render the enzyme inactive by combining with a site requiring K^+. The elucidation of the mechanism of activation and interaction of cations on this enzyme must await further investigation.

Summary

Experiments have been conducted to determine the properties and specifically the cation requirements of pyruvate kinase from certain species of higher plants. The concentration of both ADP and PEPA required for saturation of the enzyme from a dialyzed extract of pea seed were determined and from these data the appropriate Michaelis constants were estimated. After the cation requirements were established, evidence was obtained indicating that enzyme activity

was proportional to both enzyme concentration and to reaction time under the specified conditions.

Detailed studies with the enzyme from pea seed demonstrated an absolute divalent cation requirement that was satisfied by salts of Mg^{++} or Mn^{++}. The optimum concentration of these cations was 5×10^{-3} M. Co^{++} at this concentration was approximately 80 % as effective as the other two cations. The chloride salts of Ni^{++} or Ca^{++} exhibited no activating capacity and Be^{++} was only slightly stimulatory.

The enzyme from pea seed also required a univalent cation salt for maximum activity and this requirement was satisfied by the chloride salts of K^+, Rb^+ or NH_4^+. A concentration of 0.05 M resulted in maximum activity. NaCl at a concentration of 0.05 M, which was optimum, resulted in approximately 20 % of the activity obtained with a comparable concentration of K^+. The enzyme activity was essentially independent of the anion present with the exception of Br^- and I^- which were slightly inhibitory at concentrations greater than 0.05 M. An absolute requirement of univalent cations for the enzyme from pea seed was not established, however, the low activity of the preparation in absence of added univalent cations was accounted for by the content of K^+ and Na^+ in the extracts. The Michaelis constants (K_A) for the various univalent cation activators were approximately one-fifth the value reported for pyruvate kinase from rabbit muscle.

Pyruvate kinase activity was demonstrated in extracts of the seeds of wheat, cotton, oat, corn and beet; in the leaves of tobacco and peas and in the petioles of celery. The enzyme activity of extracts from all these sources was strikingly stimulated by KCl. An absolute requirement for univalent cations was demonstrated with extracts of cotton, oat and corn seed. The pyruvate kinase in those extracts, that did not show an absolute dependence on univalent cations, would not withstand dialysis periods of sufficient length to remove all endogenous univalent cations.

The authors are grateful for the competent technical assistance of Mrs. Thea Schostag.

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AN INTERFERENCE-FILTER MONOCHROMATOR SYSTEM FOR THE IRRADIATION OF BIOLOGICAL MATERIAL[1,2,3]

R. B. WITHROW

SMITHSONIAN INSTITUTION, WASHINGTON, D. C.

The determination of action spectra for biological photoreactions requires that large areas be irradiated uniformly at high intensities and with narrow band widths for the resolution of spectral details. When the material is changing irreversibly with time or when long irradiation periods are required, it may be necessary to irradiate samples at ten or more different points in the spectrum simultaneously. This excludes use of the conventional single-exit-slit monochromator and requires a spectrograph with a series of wide slits arranged in the focal plane of the spectrum. The necessity of both high radiant power and narrow band width are difficult to meet with the prism or grating spectrograph. These instruments are capable of producing band widths of a small fraction of a millimicron but the intensity of the beam falls rapidly as the waveband is decreased. Large prism (8) and grating (7) spectrograph systems using 5 to 15 kw carbon-arc sources have been described for the simultaneous irradiation of relatively large objects with monochromatic energy at many wavelengths. The band width of such systems is usually from 5 to 20 mμ when the radiant power is sufficiently high. The properties and application of these systems have been discussed elsewhere (14). The principal practical limitations of the spectrograph system are: 1) the large space required, 2) the unequal irradiance distribution over the spectrum, and 3) the cross-scattering of energy between wavelength stations. Because of the magnification required of the optical system, it is necessary to project the beam over relatively large distances of 5 to 15 meters. The relative intensity distribution between the various wavelength stations is arbitrarily determined by the spectral energy distribution of the source and transmission of the spectrograph. Therefore, it is not possible to obtain an equal irradiance or equal quantum-intensity spectrum. While this is not always essential for action spectrum studies, it is certainly desirable for convenience of analysis and interpretation of results. It is especially desirable for biological systems for which reciprocity does not hold.

The interference-filter monochromator presents several important advantages over the prism and grating instruments. A system of 10 or 20 wavelength stations can be placed in a relatively small constant-condition room. The spectral bands can be completely isolated in small cabinets and the intensity of each wavelength station adjusted independently by electrical control of the source or by neutral density filters so as to produce an equal irradiance or equal quantum-intensity spectrum. The system is simple optically, does not require high quality optical components, and is much easier to adjust than grating and prism instruments.

The interference-filter monochromator system to be described has been developed for the determination of action spectra in plants and for kinetical studies where it is desired to irradiate the material with high intensities of monochromatic energy of high spectral purity.

CHARACTERISTICS OF INTERFERENCE FILTERS

The monochromator system uses 50 mm × 50 mm second-order transmission interference filters of the Fabry-Pérot type. These filters have been discussed in several papers (4, 11) and manufacturers' brochures (1, 10). Therefore, only those properties of the filters which are of special importance in the design and use of the monochromator will be discussed here. Interference filters are now available from 340 to 1000 mμ in at least 10 mμ steps.

The transmission interference filter rejects the untransmitted energy by reflection; consequently the rejected energy is not absorbed and the filters with cemented cover glasses may be used at temperatures up to approximately 80° C. The Fabry-Pérot interference filter consists of two semi-transparent layers of silver separated by a very thin layer of a transparent dielectric material. The filter has transmission bands where the equivalent thickness of the dielectric layer is an integral multiple of a half-wavelength. Therefore, the filter has a first-order transmission band at the wavelength at which the equivalent thickness is one half-wavelength, a second-order band at two half-wavelengths, a third-order band at three half-wavelengths, and so on. The various orders of the transmission bands occur in approximate wavelength ratios of 1 : 1, 1 : 2, 1 : 3, etc. In general, the peak transmittance and the band width decrease with higher orders and, as a general compromise, most of the interference filters now available are of the second-order type which have a peak transmittance of from 20 to 45 % and a wavelength band width at half the peak transmittance (waveband half-width) of from 8 to 12 mμ in the visible spectrum, which increases to 20 mμ in the ultraviolet.

When using the second-order band of a transmission-type interference filter, it is necessary to remove the first and third orders with supplementary absorption filters. The supplementary filters must absorb transmission bands at twice the second-order wavelength on the long wavelength side and at two-thirds of the wavelength on the short wavelength side. It is desirable to select the supplementary filters with as narrow a band pass as possible because all interference filters have low transmittance of from a few

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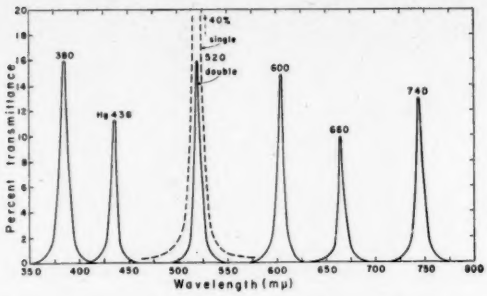

FIG. 1. Representative transmission curves of second-order interference filters. The solid curves are for two filters in tandem; the dashed curve is for one filter. The first, third and higher-order bands are not shown.

tenths to one percent in the tails between the principal transmission bands. Leakage energy presents the same problems here as does diffusely scattered energy in prism and grating instruments.

A very great improvement in spectral purity can be obtained with paired interference filters arranged in tandem. Tandem filters have tail transmissions which are almost equivalent to the products of the individual transmittances or sum of the optical densities. The tail transmittance is reduced by a factor of several hundred, although the peak transmittance is seldom decreased by a factor of more than three. The data of figure 1 were obtained for single and paired filters in a Beckman spectrophotometer. The peak transmittance varies partly because the filters were not precisely matched. Matching in dominant wavelengths is important since the transmittance curve of an interference filter is sharply peaked (fig 1) and a divergence of several mμ between filters in tandem often decreases the peak transmittance considerably below that which normally would be obtained with perfect matching. It is seldom possible to obtain filters with dominant wavelengths matched to closer than 0.3 % of the dominant wavelength, or 1 to 2 mμ. Even a 2-mμ mismatch will cause the half-width of paired filters to be equal to or larger than that of a single filter. Therefore, the principal advantage of using filters in tandem is the very great reduction in leakage of energy outside of the band pass.

LAMP SOURCES

Suitable sources include the projection-type incandescent lamps, the medium and high-pressure mercury arcs, and the zirconium, xenon, and carbon arcs (12, 14).

The most useful incandescent sources are an interchangeable group of projection lamps having medium pre-focusing bases and a distance from the base to the center of the filament structure of 2 3/16 inches. The wattage and code numbers of a series of seven 120 volt American-manufactured lamps are: 100w, 100T 8½/8; 200w, PH/200T 10P; 300w, PH/300T 10P/61; 400w, PH/400T 10P; 500w, PH/500T 10P; 750w, PH/750T 12P; and 1000w, PH/1MT 12P. The five lamps of 300w and above have biplane filaments which yield more uniform flux distribution than those with monoplane filaments. Small changes in intensity can be obtained by varying the applied voltage with a variable transformer.

As the wattage of an incandescent lamp is increased, the size of the filament structure increases correspondingly because the lamps are operated at the maximum permissible temperature for the tungsten filament. Therefore, as the wattage of the source is increased, the focal length of the condenser must be made greater in order to reduce the magnification and keep the image within the effective aperture of the filter. The increase in focal length of the condenser results in a corresponding decrease in angular aperture and the proportion of the flux that is collected by the optical system. With a specified condenser system and size of interference filter, incandescent sources larger than a certain limit produce no increase in transmitted radiant power. The limit is determined by the effective aperture of the filter and the focal length of the condenser.

In the blue and near ultraviolet, it is often advantageous to use the mercury lines. At 365, 405 and 435 mμ, a 100w mercury arc is more effective than a 500w incandescent lamp. By slightly modifying the admedium mercury arc lampholders for the 100w H-4 lamp, it is possible to use it in the same mounting as for the projection lamp. Since arcs have a negative resistance characteristic and require a minimum voltage for firing on each half of the alternating current cycle, it is necessary to introduce series resistance between the arc and the ballast. The 100-ohm rheostat shown in figure 3 makes it possible to reduce the intensity of a 100w lamp about 80 %. Further reduction in intensity produces instability of the arc and excessive damage to the cathodes.

A voltage-regulated power supply is especially important when incandescent lamps are used for the blue or ultraviolet (14). The peak of the spectral energy distribution curve of incandescent sources shifts to longer wavelengths as the temperature is reduced. A one percent reduction in voltage causes a decrease of about one percent in the near infrared, three percent in the visible and six percent in the middle ultraviolet. This radiation magnification of voltage changes can make it very difficult to balance intensities and determine incident energy values without a voltage-regulated supply.

OPTICAL SYSTEM

Unlike the prism or grating as a dispersing element, the interference filter does not require precise collimation of the incident flux. Within the limits of a 10-mμ resolution, the rays incident to the filter may diverge from the normal by as much as 15 degrees. The peak transmission shifts to the shorter wavelengths for off-axis rays, although up to 12 degrees, the shift does not exceed 6 to 8 mμ. This makes it possible to use a simple condenser without slits or

collimator optics. Short-focus, large angular-aperture condensing lenses may be used with concentrated sources in which the object distance is not less than 2.5 times the effective diameter of the lens. This gives a divergence from normal of about 13 degrees for a small concentrated source. The maximum energy is transmitted when the focal length of the condenser is such that a magnified image of the source is formed at the interference filter and the image is just large enough to include the full aperture of the filter.

The general principles of the optical system used in the interference-filter monochromator are presented diagrammatically in figure 2. This system is very similar to that of a slide projector. The condenser system, L_1 and L_2, focuses an image of the source on the projection lens, L_3. The lens, L_3, then projects an image of the condenser lens aperture in the objective plane, A′. The object to be irradiated is placed at A′ where the flux distribution is very uniform.

It is possible to dispense with L_3 and to irradiate the material with a diffused, out-of-focus image of the source, S. However, no matter how far out-of-focus the specimen is placed, some of the irregular brightness details of the source will be reproduced and the intensity distribution is not as uniform as when using a projection lens to produce an image of the condenser-lens aperture. The condenser aperture is irradiated with relative uniformity by the source. However, the center is always more intense than the edges by approximately 20 %, depending in part upon the source distance, f_1. If extremely uniform flux distribution is desired, it is necessary to use a long focal-length condenser lens. The source is relatively far removed from the condenser and the condenser aperture more uniformly irradiated. A larger wattage lamp is then required to produce the same transmitted flux.

The interference filters are placed as close to the objective lens, S′, as possible. In the case of the Bausch and Lomb interference filters, which are approximately 50 mm square, the useful aperture is only 38 mm. The filters are not aluminized over the whole face of the glass plate, in order to leave a small margin of unsilvered glass for bonding the cover glass. The filter should be blackened on the edges and on the margins of both faces for about 6 mm to prevent scattering of unfiltered energy around the edges of the silver-dielectric film (fig 2).

A primary aqueous filter, F_1, is used between the

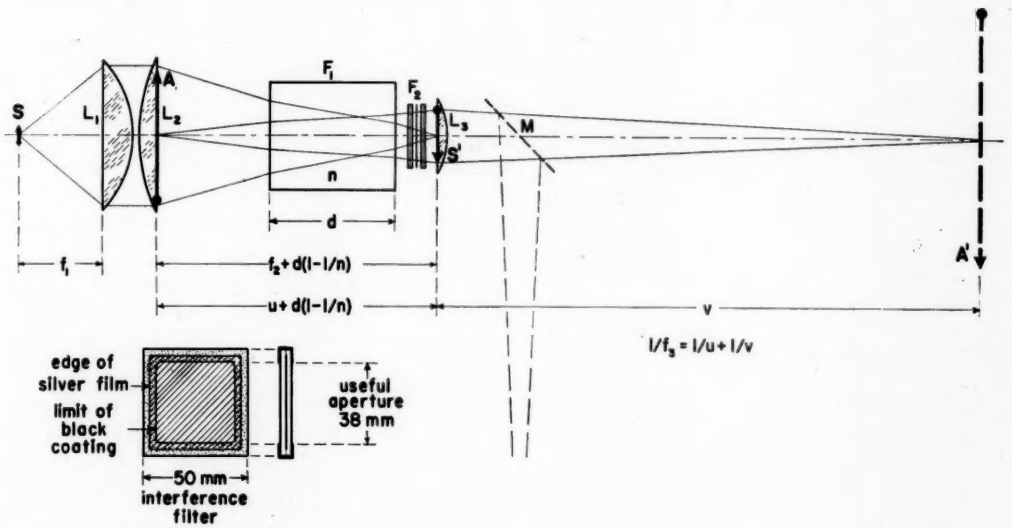

FIG. 2. Schematic diagram of the optical system of a transmission interference-filter monochromator unit.

Legend:

S—Source
S′—Image of source produced by condenser system L_1 and L_2 of focal lengths f_1 and f_2 respectively
F_1—Aqueous filter of refractive index, n, and path length, d. This filter increases the actual object distance by $d(1-1/n)$.
F_2—Assembly of two interference and one dyed-gelatin filters
L_3—Projection lens of focal length, f_3
M—Mirror
A′—Magnified image of uniformly irradiated aperture, A, at position of condenser lens, L_2
u—Object distance of L_3
v—Image distance of L_3

condenser and the interference filter, F_2 (13). Because of the relatively great depth, d, of this filter (10 cm in fig 3), the effective image distance is appreciably increased (6). Formulas used for calculating the image distances are given in figure 2. It will be noted that the increase in effective distance between the condenser lens and the source image is a function of the refractive index of the filter solution which is approximately 1.3 for aqueous filters. The upper formula gives the increase in apparent focal length of L_2 in a two-lens condenser. The lower formula gives the increase in objective distance which would be obtained if a single condenser lens is used.

Monochromator Unit

The complete details of a single monochromator unit with associated electrical circuitry and cabinet are given in figure 3. In our laboratories we have ten of these units mounted on a long table in a constant-condition darkroom. They are powered from a single voltage regulator and the whole system is controlled by a time switch. The ten-station system is designed for the irradiation of small leaves and stems. These are arranged in a circle in a 150-mm-diameter dish, D, centered on the emergent beam.

The filter system consists of four or five components, depending upon the region to be investigated. The first supplemental filter, F_1, is water or an aqueous solution of ferrous ammonium sulfate or copper sulfate, in an all-glass filter cell of 10 cm internal length. The properties of these filters have been discussed (2, 5, 9, 13, 14). This filter is cooled by a small hairpin loop of ⅛ inch silver tubing. Fine silver is used because it is very ductile and not reacted upon by salts of copper and iron in the presence of free acid. The interference filters, F_2 and F_4, and the gelatin filter, F_3, (3, 13) are mounted in a half-inch aluminum plate. The neutral density filter, NF, is useful for obtaining very low irradiances.

The filter, F_5, absorbs stray flux and is only of value under certain conditions. For determination of a red-sensitive response in the green, blue and ultraviolet, the filter, F_5, is a solution of copper sulfate. For a blue-sensitive biological system, a yellow filter is required. The window, F_6, is essentially a far-infrared-absorbing filter of lucite and prevents air, circulating from the blower, BL, from entering the irradiation cabinet. Warm components produce long-wavelength infrared which can cause appreciable deflection of a thermopile and often is confused with the energy transmitted by the filters. This annoying property is practically eliminated by a ¼ inch plate of methacrylate plastic such as plexiglas or lucite (14).

A listing of the supplemental filters for use with tandem interference filters is given in table I. For wavelengths longer than 520 mμ, long-wave-pass gelatin filters with sharp cut-off are indicated, with band pass filters specified for the shorter wavelengths. In general, the selection of supplemental filter combinations was made so as to produce as narrow a band pass as possible, consistent with reasonably high transmittance.

With two interference filters and a 750w lamp, the monochromator of figure 3 will irradiate an object 20 cm in diameter with 5 to 20 μw/cm² at any point in the spectrum from 365 to 800 mμ and with a single filter, 20 to 100 μw/cm². A larger monochromator unit has been designed for a 7-kw condenser-type rotating carbon arc. It employs 6-inch diameter condenser lenses. This system produces irradiances in the red of 300 μw/cm² with double filters and up to 2000 μw/cm² with a single filter. When properly adjusted, both the small and large units produce a variation of about ± 10 % in intensity over the 20-cm diameter aperture. By arranging the material to be irradiated in a circular pattern, the variation in irradiance can be kept very small. The spectral resolution of the system is adequate for resolving spectral details separated by 10 mμ. With careful selection

Table I

Monochromator Filter Systems

Interference, mμ **	Gelatin or glass no.†	Aqueous, 10 cm pathlength ††	
		Salt	Conc, gm/l
365 (Hg)	18A	Cu	200
385	18A	Cu	200
405 (Hg)	3 *	Cu	200
420	3 *	Cu	200
436 (Hg)	4 *	Cu	200
450	4 *	Cu	200
460	4 *	Cu	200
480	40	Cu	200
500	57	Cu	100
520	61	Cu	100
530	15	Cu	100
546 (Hg)	16	Cu	50
560	16	Cu	20
578 (Hg)	23A	Cu	20
600	26	Cu	10
610	26	Cu	10
620	26	Cu	10
630	26	Fe	150
640	29	Fe	150
650	29	Fe	150
660	2 × 29	Fe	150
670	70	Fe	150
680	70	Fe	75
690	70	Fe	75
700	89	Fe	75
710	89B	Fe	75
720	89B	Fe	75
730	88	Fe	38
740	88A	Fe	38
750	88A	Fe	38
760	88A	Fe	38
780	87	Water only	
800	87	Water only	

** Incandescent lamps used as sources except as noted for mercury arc, Hg.

† Kodak Wratten No. 3 except for those designated *, see (13).

†† Cu = $CuSO_4 \cdot 5\,H_2O$. Fe = $Fe(NH_4)_2(SO_4)_2 \cdot 6\,H_2O$. Salts prepared in 1 % sulfuric acid.

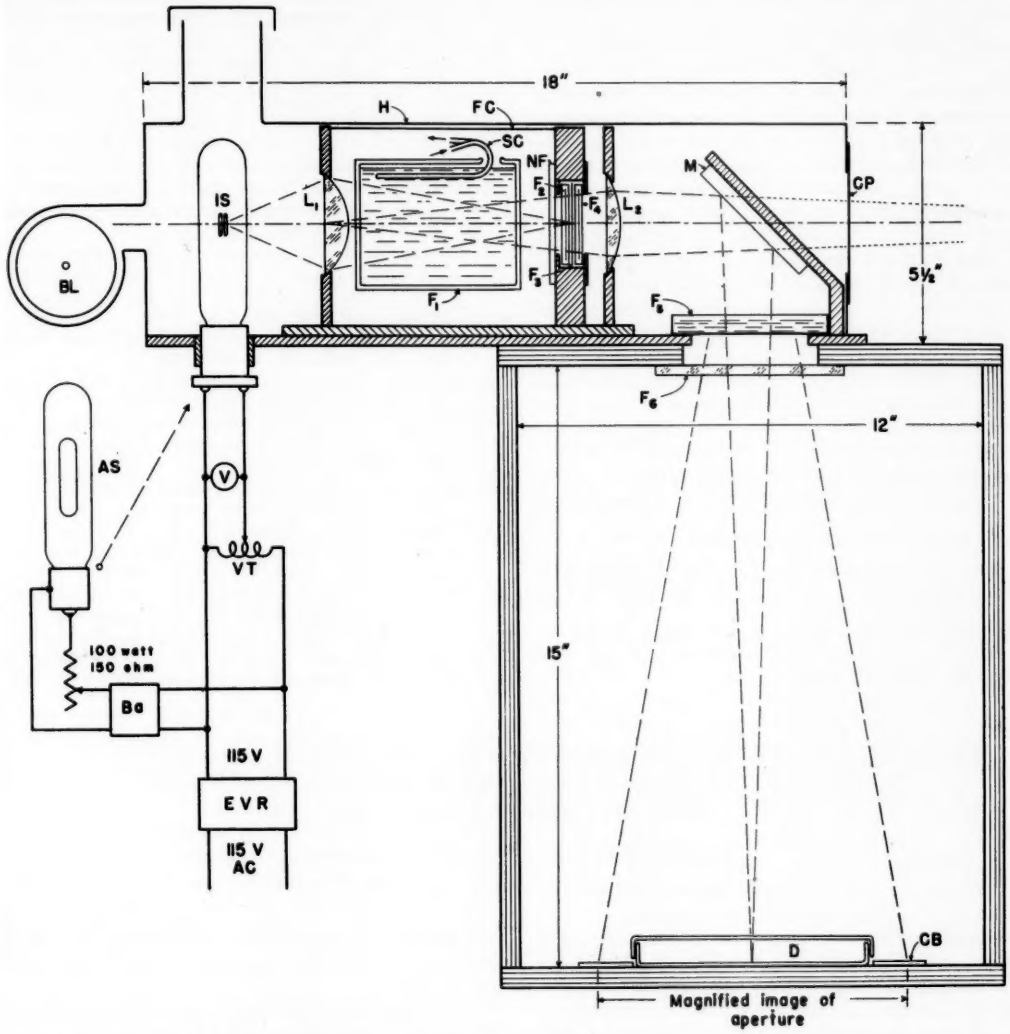

FIG. 3. Diagram of a single interference-filter monochromator mounted on exposure cabinet. Control circuits are shown for both incandescent and mercury arc lamps.

Legend:

IS—Incandescent source, 100-1000-watt projection lamp
AS—Arc source, 100-watt, H100-A4 type mercury arc
BL—Blower
L_1—Condenser lens, 65 mm diam, 50 mm focus
L_2—Projection lens, 65 mm diam, 80 mm focus
F_1—Aqueous filter, 10 cm path length
F_2, F_4—Second-order, interference filters, 50 mm × 50 mm
F_3—Dyed-gelatin filter
F_5—Secondary aqueous filter for removing scattered flux
F_6—Secondary infrared filter of ¼ in. methacrylate plastic
NF—Neutral filter
FC—Filter cell inner cover
SC—Silver cooling coil
M—Plane mirror
H—Removable housing
D—Petri dish, 150 mm diam
CB—Centering board
CP—Cover plate
V—Voltmeter
VT—Variable transformer
EVR—Electronic voltage regulator
Ba—Mercury arc lamp ballast
R—Rheostat, 150 ohms, 150 watts

of filters, it might be possible to obtain 5 mμ resolution.

Summary

The design of a small compact interference-filter monochromator system for the spectral range from 365 to 800 mμ is presented, together with a theoretical discussion of optical principles. The characteristics of interference filters and supplemental gelatin and aqueous filters are given. The monochromator has a resolution of about 10 mμ throughout the visible spectrum and can produce relatively high irradiances with an incandescent lamp. Irradiances as high as 2000 μw/cm² can be secured with a larger unit employing a 7-kw carbon arc.

The author wishes to express appreciation to Dr. W. H. Klein and Mr. V. Elstad who made many valuable suggestions for modifications while using the monochromator system, and to Mr. D. G. Talbert who constructed the instrument and Mr. J. H. Harrison who assisted in the design of the optical system.

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THE RELATION OF OPTICAL FORM TO THE UTILIZATION OF AMINO ACIDS. I. UTILIZATION OF STEREOISOMERIC FORMS OF GLUTAMIC ACID BY CARROT ROOT DISKS[1]

E. D. H. EL-SHISHINY and M. A. NOSSEIR

Botany Department, Faculty of Science, Alexandria University, Egypt

According to the classical idea of Liebig the plants are able only of assimilating inorganic nitrogen. But now there is no longer reason to doubt the ability of plants to absorb and utilize organic nitrogenous compounds, since in sterile culture plants can utilize various organic nitrogenous compounds, amino acids and amides. The extensive early literature describing the utilization of organic nitrogenous compounds by plants has been reviewed by Hutchinson and Miller (7) and by Brigham (2). Several other reports concerning the availability of amino acids for plant growth, that followed these papers, were reviewed by Ghosh and Burris (6).

Very little experimental work was carried out on the relation of optical form to the utilization of amino acids in higher plants, and our knowledge in this field is very scanty. The utilization of both optical forms of aspartic and glutamic acids by pea and clover was reported by Virtanen and Linkola (15). The synthesis of both isomers of glutamine from their respective isomers of glutamic acid catalysed by a purified enzyme from pea was shown by Levintow and Meister (9, 10).

The purpose of this investigation is to compare the ability of carrot root cells to absorb and assimilate the various stereoisomeric forms of glutamic acid and to attempt some explanations of the results obtained.

Material, Methods, and Experiments

The disks for this investigation were prepared from carrot roots var. Chantenay. The general procedure of preparation and pre-treatment of the disks as well as the determination of the nitrogenous fractions were as described by El-Shishiny (4). Twenty grams of disks, taken at random from a stock of disks prepared for each experiment, were used for each sample. The samples were washed for four days in aerated distilled water. The samples were then washed several times with sterilized distilled water and transferred into the sterile culture solutions, kept at 25° C (± 0.1°). The samples were aerated for

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24 hours by passing CO_2-free air through each culture solution at a constant rate of four liters per hour. The air currents, after leaving the culture chambers, were passed through standard solutions of sodium hydroxide to determine the rates of CO_2 production by the different treated tissues. After 24 hours the tissue samples were drained, washed with distilled water, and analysed for the final distribution of the various nitrogenous fractions. The medium, together with the washings, was made to a convenient volume and analysed for inorganic and organic nitrogen fractions. The initial distribution of various nitrogenous fractions in the tissues was obtained by analysing two samples representing the batch of disks after the four days washing period.

Nitrogen fractions:

Protein-N = insoluble-N + nitrogen fraction precipitated with 2.5 % trichloroacetic acid.

Non-protein-N = total soluble-N – nitrogen fraction precipitated 2.5 % trichloroacetic acid.

Amino acid-N = total amino-N – amino-N equivalent to amide-N.

Rest-N = non-protein-N – (inorganic-N + total amino-N + amide-N).

Complex-N = protein-N + rest-N.

Glutamine-N = 2 × glutamine amide-N.

Asparagine-N = 2 × asparagine amide-N.

Experiment I was designed to investigate the uptake and utilization of L-glutamic acids when supplied alone and in combination with ammonium sulphate to carrot root disks. Twelve samples after being washed for 4 days, were transferred into culture vessels, each containing 350 ml of sterile culture solution, according to the following scheme: Samples 1 and 2, distilled water; 3 and 4, 0.0025 M ammonium sulphate; 5 and 6, 0.0025 M L-glutamate; 7 and 8, 0.0025 M L-glutamate + 0.0025 M ammonium sulphate; 9 and 10, 0.0025 M D-glutamate; 11 and 12, 0.0025 M D-glutamate + 0.0025 M ammonium sulphate. The results obtained from this experiment were recorded in table I and III.

TABLE I

AMINO ACID AND AMMONIUM NITROGEN ABSORBED AND ASSIMILATED BY CARROT ROOT DISKS DURING 24 HOURS FROM DIFFERENT CULTURE SOLUTIONS

CULTURE MEDIA	MG N*/100 GM FRESH WT OF TISSUE			
	ABSORBED		ASSIMILATED	
	NH_4 – N	AMINO ACID-N	NH_4 – N	AMINO ACID-N
Distilled water	0.00	0.00	– 0.32	– 0.41
0.0025 M $(NH_4)_2SO_4$	45.33	0.00	34.77	1.37
0.0025 M L-Glutamate	0.00	38.41	– 0.65	27.10
0.0025 M $(NH_4)_2SO_4$ + 0.0025 M L-Glutamate	68.14	38.90	57.52	36.93
0.0025 M D-Glutamate	0.00	13.59	– 0.32	3.55
0.0025 M $(NH_4)_2SO_4$ + 0.0025 M D-Glutamate	23.57	14.65	8.12	5.60

* Means of duplicate samples.

TABLE II

AMINO ACID AND AMMONIUM NITROGEN ABSORBED AND ASSIMILATED BY CARROT ROOT DISKS DURING 24 HOURS FROM DIFFERENT CULTURE SOLUTIONS

CULTURE MEDIA **	MG N*/100 GM FRESH WT OF TISSUE			
	ABSORBED		ASSIMILATED	
	NH_4 – N	AMINO ACID-N	NH_4 – N	AMINO ACID-N
0.0025 M $(NH_4)_2SO_4$	40.11	0.00	29.88	2.57
0.0025 M L-Glutamate	58.71	28.76	48.60	29.41
0.005 M L-Glutamate	70.90	46.07	60.52	47.19
0.0025 M L- + 0.0025 M D-Glutamate	44.00	38.42	28.35	34.83
0.0025 M L- + 0.005 M D-Glutamate	42.20	44.10	26.87	37.60
0.005 M DL-Glutamate	40.42	43.67	24.45	39.20

* Means of duplicate samples.
** All media contain 0.0025 M $(NH_4)_2SO_4$.

Experiment II was designed to investigate the effect of D-glutamate on the uptake and utilization of the other enantiomorph when supplied simultaneously to the tissues. Twelve samples, after being washed for 4 days, were transferred into culture chambers, each containing 350 ml of sterile culture solution, according to the following scheme: Samples 1 and 2, 0.0025 M ammonium sulphate; 3 and 4, 0.0025 M ammonium sulphate + 0.0025 M L-glutamate; 5 and 6, 0.0025 M ammonium sulphate + 0.005 M L-glutamate; 7 and 8, 0.0025 M ammonium sulphate + 0.0025 M L-glutamate + 0.0025 M D-glutamate; 9 and 10, 0.0025 M ammonium sulphate + 0.0025 M L-glutamate + 0.005 M D-glutamate; 11 and 12, 0.0025 M ammonium sulphate + 0.005 M DL-glutamate. The results obtained from this experiment were recorded in tables II and IV.

L-, D-, and DL-Glutamic acid of CEP grade were purchased from the California Foundation for Biochemical Research. The other chemicals used were of analytical grade produced by Merck and Company, Inc. The amino acids were neutralized to pH 7 with KOH before supplying them to the tissues.

RESULTS AND DISCUSSION

UPTAKE AND ASSIMILATION OF L- AND D-GLUTAMIC ACIDS: It appears from the results depicted in table I that carrot root disks have absorbed far more L- than D-glutamate-N from their respective solutions. The absolute amount of amino-N absorbed from L-glutamate was about three times larger than that absorbed from D-glutamate culture solutions. Glutamate uptake was accompanied with higher respiration rates. This seemed to indicate the dependence of glutamate uptake upon respiratory energy as suggested by Webster (17). But differences in the degree of

absorption of the two isomers may be the result of differences in their rates of penetration into the cell. It may be suggested that the amino acid first combines with some cellular constituent at the surface and this complex is transported into the cell. Since the membrane consists of optically active material, differences in penetrability of stereoisomers according to this line might be possible.

Carrot root disks showed marked ability to utilize L-glutamic acid since the deficit in the balance of amino acid-N was equivalent to 70.6 % of the absorbed amino-N from the L-glutamate medium, table I. The utilization of glutamic acid by other plant tissues was also reported by other investigators (1, 3, 5, 12, 13, 14, 15 and 17). The presence of ammonium sulphate with L-glutamate in the culture solution promoted the utilization of the L-glutamate. The stimulation here was the result of the increased ammonium-N level in the cells, since the amonut of amino-N absorbed from L-glutamate was equal to that absorbed from L-glutamate-ammonium sulphate medium. But the increase of L-glutamate concentration in the L-glutamate-ammonium sulphate medium; from 0.0025 M to 0.005 M, promoted both the absorption and assimilation of amino acid-N to the same extent, table II.

On the contrary, there was no convincing evidence that carrot root disks were able to utilize D-glutamic acid. The deficits in the balance of amino acid-N in carrot disks cultured in solutions of D-glutamate alone or with ammonium sulphate were very small. However, when D-glutamate was added to the L-glutamate–ammonium sulphate medium, or when DL-glutamate was used simultaneously with ammonium sulphate, table II, more amino acid-N was utilized. But judging by the results obtained from experiment I, table I, and the demonstrated synergistic effects of mixtures of stereoisomers of amino acids by Macht (11), this increase might be on the expense of the L-form absorbed and that originally present in the cells. And since the method used for the determination of amino acid-N did not differentiate between the two isomers, the above suggestion needs confirmation, and therefore awaits further investigation. Results obtained by other investigators showed that the ability to utilize D-glutamic acid varies greatly for the various plants. Virtanen and Linkola (15) reported that pea and clover utilize both forms of glutamic acid, while Levintow and Meister (10) demonstrated that the rate of glutamine synthesis from D-glutamate is about 40 % of the rate of the L-isomer catalysed by an enzyme preparation from peas.

EFFECT OF L- AND D-GLUTAMATE ON THE UPTAKE AND UTILIZATION OF AMMONIUM-N: As shown in tables I and II, ammonium and L-glutamate, when present together in the outer solution, were absorbed simultaneously by carrot root disks, but ammonium in preference to glutamate.

It was also shown that L-glutamate, when present simultaneously with ammonium sulphate in the culture solution, enhanced greatly the total uptake of ammonium-N. On the contrary D-glutamate, under similar conditions, exerted a marked depressing effect on the uptake of ammonium-N. Moreover when the concentration of L-glutamate, in the L-glutamate–ammonium sulphate medium, was doubled the total uptake of ammonium-N was increased by 76.8 %. But when the increase in glutamate concentration, in the L-glutamate–ammonium sulphate medium, was due to the antipode, the stimulating effect of L-glutamate on ammonium-N uptake was neutralized by the inhibiting effect of the antipode. The total amount of ammonium-N absorbed was more or less equivalent to the amount absorbed from the control solution of ammonium sulphate alone.

L-glutamate stimulated ammonium assimilation which appeared to be the direct effect of the increased absorption of ammonium-N, since the absolute increase in ammonium assimilation due to L-glutamate was equivalent to the absolute increase in ammonium-N absorption. This result accounts for the equivalent levels in ammonium-N in tissues cultured in ammonium sulphate and in ammonium-sulphate-L-glu-

TABLE III

EFFECT OF CULTURING CARROT ROOT DISKS IN SOLUTIONS OF 0.0025 M L- OR D-GLUTAMIC ACID ALONE OR IN COMBINATION WITH 0.0025 M AMMONIUM SULFATE

NITROGEN FRACTIONS	CONTROLS *		CHANGES IN N FRACTIONS DURING 24 HRS			
			H_2O **		$(NH_4)_2SO_4$ †	
	H_2O	$(NH_4)_2SO_4$**	L-GLUTAMATE	D-GLUTAMATE	L-GLUTAMATE	D-GLUTAMATE
	mg N/100 gm initial fresh wt of tissue ††					
Ammonia-N	0.32	+10.24	+ 0.33	0.00	+ 0.06	+ 4.89
Glutamine-N	1.91	+15.82	+ 6.49	+1.96	+42.51	−11.68
Asparagine-N	0.32	+ 4.96	+ 4.52	+2.26	+ 7.08	+ 0.13
Amino acid-N	8.67	− 1.78	+10.90	+9.63	+ 3.34	+10.42
Rest-N	5.52	+ 3.38	− 2.89	−2.90	− 0.49	− 5.45
Protein-N	66.78	+11.80	+16.95	+2.61	+ 8.72	− 6.32

* Control tissues are cultured in distilled water and in 0.0025 M ammonium sulfate.
** Compared with control in water.
† Compared with control in ammonium sulfate.
†† Means of duplicate samples.

TABLE IV

EFFECT OF CULTURING CARROT ROOT DISKS IN SOLUTIONS OF 0.0025 M AMMONIUM SULFATE ALONE OR IN COMBINATION WITH L-, L- AND D-, OR DL-GLUTAMIC ACID

NITROGEN FRACTIONS	CONTROL IN $(NH_4)_2SO_4$	CHANGES IN THE N FRACTIONS DURING 24 HRS *				
		0.0025 M L-GLUTAMATE	0.005 M L-GLUTAMATE	0.0025 M L- AND 0.0025 M D-GLUTAMATE	0.0025 M L- AND 0.005 M D-GLUTAMATE	0.005 M DL-GLUTAMATE
		mg N/100 gm initial fresh wt of tissue **				
Ammonia-N	10.99	− 0.12	+ 0.15	+ 5.42	+ 5.10	+ 5.74
Glutamine-N	19.33	+34.65	+54.39	+24.91	+22.82	+25.19
Asparagine-N	6.06	+ 2.19	+ 7.01	+ 2.72	+ 3.52	+ 2.80
Amino acid-N	5.31	+ 1.92	+ 1.45	+ 6.16	+ 9.07	+ 7.04
Rest-N	7.00	+ 5.50	+11.65	+ 5.16	+ 3.67	+ 5.16
Protein-N	63.79	+ 2.65	+ 0.01	− 4.66	+ 1.03	− 1.21

* Compared with control in ammonium sulfate.
** Means of duplicate samples.

tamate media, tables III and IV. On the contrary, D-glutamate expressed greater depressing effects on the assimilation than on the absorption of ammonium-N by carrot root disks, table I. Even when D-glutamate was supplied simultaneously with L-glutamate and ammonium sulphate, the absolute amounts of ammonium-N assimilated were less than that with ammonium sulphate alone. Consequently the ammonium-N levels in tissues cultured in solutions containing D-glutamate were always higher than the level of ammonium-N in tissues cultured in ammonium sulphate alone.

EFFECT OF L- AND D-GLUTAMIC ACID ON THE DISTRIBUTION OF THE VARIOUS NITROGENOUS FRACTIONS: The increase in the levels of amide-N and complex-N in tissues cultured in solutions containing L-glutamate alone or with ammonium sulphate, over those of control tissues in distilled water and ammonium sulphate respectively, tables III and IV, suggested the utilization of L-glutamate in the formation of these fractions. In the absence of ammonia, it seems probable that one part of L-glutamate absorbed was deaminated and that the ammonia produced was involved in amidation of the remaining part as suggested by Krebs (8) and Webster (16). The results of the present investigation indicated that ammonia was removed as fast as it was released since the tissues did not show any significant change in the levels of ammonium-N. Moreover the tissues cultured in L-glutamate alone table III, showed greater increase in the protein than in the amides. This might indicate the incorporation of glutamic acid in protein formation. The incorporation of a significant amount of C^{14} of L-glutamate in the tissue proteins of carrot and other plant tissues was proved by Webster (17).

On the other hand, tissues cultured in D-glutamate alone, table III, showed an increase of 4.22 mg amide-N × 2 and a deficit in the balance of amino acid-N of 3.55 mg per 100 gm fresh weight of carrot disks. And since the increase in protein-N balances the decrease in rest-N, it might be concluded that D-glutamate was involved in the formation of amides. But the increase in amide-N might be on the expense of the natural form originally present, since addition of D-glutamate to ammonium sulphate caused a marked decrease in the amide-N × 2 and complex-N below that of control disks cultured in ammonium sulphate alone. The latter conclusion was again substantiated by the results of experiment II, table IV. These results indicated that D-glutamate exerted a depressing effect on the formation of amides from L-glutamate and ammonia. The degree of inhibition of the glutamine sythesizing system in carrot root by D-glutamate was less than that reported by Krebs (8) for that of guinea pig kidney.

SUMMARY

The uptake and utilization of the different stereoisomers of glutamic acid by carrot root disks were studied.

1. L-glutamate was absorbed at a much faster rate than the D-enantiomorph.

2. Carrot root disks showed a marked ability to utilize L-glutamate especially when supplied in combination with ammonium sulfate. On the other hand, there is no evidence of the utilization of D-glutamate which seems to accumulate in the cells.

3. L-glutamate stimulated the rate of ammonium uptake and assimilation in contrast to the depressing effect of D-glutamate on both uptake and assimilation of ammonium-N.

4. Addition of D-glutamate to L-glutamate neutralized the stimulating effect of the latter on the uptake and utilization of ammonium-N.

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THE INFLUENCE OF BORON ON STARCH PHOSPHORYLASE AND ITS SIGNIFICANCE IN TRANSLOCATION OF SUGARS IN PLANTS[1,2]

W. M. DUGGER, JR., T. E. HUMPHREYS and BARBARA CALHOUN
Department of Botany, Florida Agricultural Experiment Station,
University of Florida, Gainesville, Florida

An increase in the translocation of sugars in plants caused by boron has been demonstrated by Gauch and Dugger (6) and Sisler et al (26). Mitchell et al (22) obtained additional evidence that boron increases translocation of sugars and indirectly the translocation of growth-modifying substances. Previous work by Mitchell and Brown (21) and others showed that translocation of growth-modifying substances from leaves to other plant parts was dependent upon the translocation of sugars.

The hypothesis presented by Gauch and Dugger (6) to explain the influence of boron on translocation is based on: 1) the formation of borate-sugar complex; and 2) the movement of the complex through cellular membranes more rapidly than non-borate sugar molecules. Of the several possible explanations for the way boron could cause an increase in sugar translocation, these investigators favored the one which associated the borate ion with the cellular membrane. In this position the borate ion reacts chemically with the sugar molecule, and the resulting complex is transported across the membrane. A second reaction on the inside of the membrane releases the sugar into the cell. Because of the rapid utilization of labeled sugars by living cells, these workers did not investigate this "carrier" concept as has been done in mineral element absorption studies (4).

Plants grown in a medium deficient in boron have been shown to accumulate carbohydrates, particularly starch, in the leaves (7). Because of this effect and the studies which show that boron increases translocation, it was decided to study the influence of boron on starch formation in vivo and in vitro.

In vitro studies by Winfield (29) showed a slight inhibition of starch phosphorylase by high concentrations of boron. He concluded that boron reacted with sugars in the same way as phosphorus; and because of the small boron:phosphorus ratio in plants, he did not believe that boron influenced the starch $\rightleftarrows$ glucose-1-phosphate equilibrium. Torssell (27), however, found no effect of 10^{-3} M arylboric acids on starch phosphorylase.

The effect of boron on the starch phosphorylase reaction has been reinvestigated in view of the fact that boron may possibly complex glucose-1-phosphate and thus prevent its conversion to starch. In addition the influence of boron on the hexokinase reaction has also been investigated. The results of these studies are presented in this paper.

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Methods and Results

Ten-day-old snap bean plants, *Phaseolus vulgaris* L. var. Black Valentine, were transferred from the greenhouse to a dark room. After 48 hours in the dark, the level of starch in the primary leaves was reduced from 12 mg/gm fresh weight to 3 mg/gm fresh weight. At this time the leaves were removed, and 2- to 3-gram samples were rapidly weighed to the nearest mg and submerged in a solution of 4 % glucose containing 0.001 % Tween-20 (Polyoxyethylene sorbitan monolaurate) and 0.2 ml of 1 M K_2HPO_4 per 100 ml. Other leaf samples were submerged in a solution containing various levels of boron, glucose, Tween-20 and phosphate. The leaf material was then subjected to vacuum infiltration according to McCready and Hassid (20). After infiltration, the leaves were removed and washed three times in tap water and twice in distilled water. They were then placed in covered dishes on moist filter paper and stored for 24 hours in the dark at 25° C. At this time the leaves were removed from the dishes and used in one of three analytical procedures: (a) starch analysis, (b) boron analysis, (c) respiration studies. The starch content was determined by digesting the alcohol insoluble residue with taka-diastase for 24 hours and subsequent acid hydrolysis to form glucose (1). Reducing sugars were determined by the method of Nelson (23). The colorimetric method of Dible et al (3) was used to analyze the plant tissue for boron content.

Table I shows the influence of boron on the starch content of these leaves. There is a definite decrease in the amount of starch synthesized by the leaves when infiltrated with solutions containing glucose and 20 millimoles/l or more of boron. It appears from these data that the addition of boron, at least above 10 millimoles/l, brings about some change in the metabolism of the leaf material that influences the synthesis of starch from added glucose. If this is associated with the conversion of glucose-1-phosphate to starch by starch phosphorylase then the boron is inhibiting the reaction in some manner.

Table I

The Influence of Boron on the Starch Synthesis in Black Valentine Bean Leaves Infiltrated with 4 % Glucose and Various Levels of Boron (Av of 6 expts) *

B in infiltrating solution, millimoles/l	Starch as red. sugar, mg/gm fresh wt
Water	2.3
Glucose	3.9
1	3.9
5	3.9
10	3.6
20	2.9
50	2.6
100	2.8
L.S.D. 5 %	1.1
L.S.D. 1 %	1.4

* The analysis was determined at 24 hrs after the leaves were infiltrated and placed in moist chambers in the dark at 25° C.

Table II

The Influence of Infiltrating Black Valentine Bean Leaf Tissue with Various Concentrations of Boron on the Analysis of Boron

B in infiltrating solution, millimoles/l	B in solution, μgm/gm	B in tissue, μgm/gm fresh wt	Ratio, solution/tissue
1	10	1 ± 0.5	10
5	50	7 ± 3	7
10	100	15 ± 4	7
20	200	20 ± 5	10
50	500	52 ± 0.5	10
100	1000	66 ± 6	15 (Av 9.8)

Because of the high levels of boron used in the infiltrating solutions, it was thought that the results might be caused by a toxicity effect on the overall metabolism (14). The analysis of the leaf material for boron was carried out with three of the experiments. The results are given in table II. Column 2 of this table is obtained by multiplying the values in the first column by 10 (the atomic weight of boron). In this way the analytical values in column 3 can be compared to the amounts in solution. This comparison is given in column 4 of the table. Only about one-tenth the concentration of boron used in the solution for infiltration remains on or in the leaves after five washings with water. Of this one-tenth, the amount actually entering the cells and influencing physiological processes is surely much smaller. In these experiments where starch synthesis is first reduced by a significant amount, the actual boron concentration in the bean leaves is below the values given by Dible et al (3), for non-toxic levels in plants.

Additional evidence for assuming that non-toxic levels of boron were present is given in figure 1. These curves represent the oxygen uptake by leaf disks from 24 to 30 hours after infiltration with glucose and boron. There is a definite maximum response at 5 and 10 millimoles of boron per liter in the infiltrating solution; but in all concentrations used, the total oxygen uptake by the bean leaf disks, pre-treated with boron in the infiltrating solutions, was greater than that of the control disks pre-treated with glucose alone. The endogenous respiration of disks cut from water infiltrated leaves has been subtracted from all values. This experiment was repeated three times, and the same conclusions were apparent. It was also noted in all 12 experiments in which bean leaves were infiltrated with glucose and boron solutions that the leaves were turgid and appeared healthy 24 hours after infiltration. Even though high levels of boron were used in the infiltrating solutions, the effective levels are within physiological range.

An accumulation of carbohydrates in leaves of plants deficient in boron has been reported by many investigators as cited by Gauch and Dugger (7). It

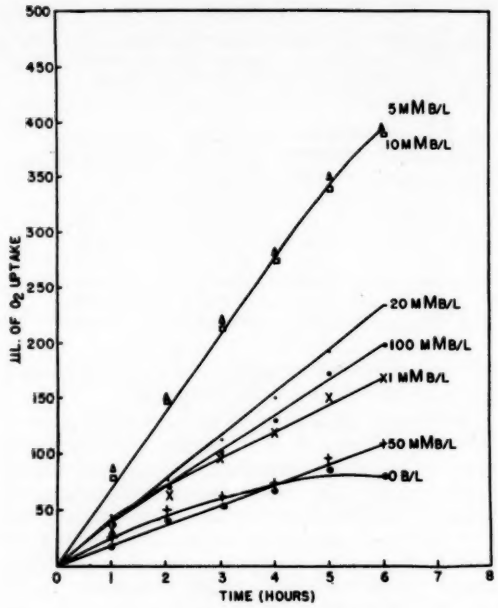

FIG. 1. The respiration of bean leaf disks 24 to 30 hrs after infiltration with 4 % glucose and various levels of boron. Fifteen disks per vessel. All treatments were duplicated.

would therefore seem logical that the addition of boron to leaves adequately supplied with boron, as described in the previous experiments, would result in a decrease in starch content if the mechanism of starch synthesis is altered by the borate ion. On the other hand, if the boron were acting as a carrier and were facilitating translocation, there should have been no difference in the starch content of the detached leaves infiltrated with glucose and boron as compared to glucose alone. However, the data presented do not negate the other hypothetical types of action previously mentioned. It was thought that an in vitro study might shed additional light on this problem.

Starch phosphorylase was obtained by the method of Hanes (12) from white potato. This procedure involves a fractional centrifugation of the 0.3 to 0.5 saturated ammonium sulfate precipitate. Tests of the extract for phosphatase and amylase indicated the absence of these enzymes. The preparation did contain a small amount of amylose, but not sufficient to prime the reaction (glucose-1-phosphate $\rightleftarrows$ starch + phosphate) at a maximum rate. Additional starch was added to prevent this factor from being limiting. One ml of enzyme was mixed with 0.2 ml of a 5 % starch solution, 0.5 ml of sodium citrate buffer (pH 6.0) and water or boric acid to make a total volume of 2.5 ml. After equilibrating at 35° C, one ml of a 0.1 M glucose-1-phosphate solution was added. This digest mixture, as given by Green and Stumpf (10), was the basis for variation in components during the course of the experiments.

The reaction was measured in two ways. The one more often used was to transfer a one-ml aliquot of the digest to five ml of a 6 % trichloroacetic acid solution. After several minutes the acid was neutralized with 6 % ammonium hydroxide and the inorganic phosphorus was determined by a modified Fiske and SubbaRow method (5). In some experiments the reaction was measured by determining the amount of starch formed. This method of Hanes and Cattle (13) involves the development of a starch-iodine color. The optical density is determined at 700 mμ and values are compared to a standard curve. Figure 2 indicated the kind of results obtained in the early experiments. However, the degree of response to boron was different between experiments and the influence of the ion on the reaction varied from 30 % inhibition (fig. 2) to complete inhibition by 50 millimoles/l of boron in several experiments.

To show that the influence of boron on the glucose-1-phosphate conversion to starch is on the rate of reaction and not on the final equilibrium value, the experiment reported in table III is presented. By comparing column 4 and 5 of this table, it is seen that when 10 millimoles/l of boron is present in the digest, the rate of liberation of inorganic phosphate, and evidently starch synthesis, is slower. The equilibrium value for the ratio of inorganic phosphorus to total phosphorus, however, is the same: 0.865. Hanes (12) reported an equilibrium value of 0.87 for this reaction at pH 6.0.

Because the hydrogen ion concentration influences the equilibrium value and rate of reaction, it is necessary to measure pH along with the reaction products. Columns 2 and 3 indicate that there was a shift in pH over the course of reaching an equilibrium, but in no case was the difference in pH between no boron and 10 millimoles/l of boron greater than 0.1 pH units. In view of Hanes's very extensive report on starch phosphorylase, it was apparent that the observed ef-

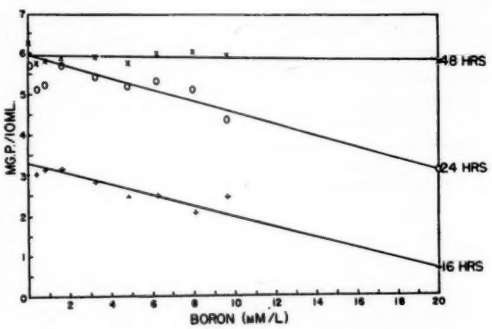

FIG. 2. The influence of boron on the starch phosphorylase enzymatic synthesis of starch from glucose-1-phosphate. Aliquots of the reaction mixtures were taken for analysis at the times shown.

TABLE III

THE INFLUENCE OF BORON ON THE RATE GLUCOSE-1-PHOSPHATE IS CONVERTED TO STARCH BY POTATO STARCH PHOSPHORYLASE ENZYME (AV OF 2 EXPTS)

HRS FROM START	pH		MG P/10 ML DIGEST	
	- B	+ B, 10 MILLIMOLES/L	- B	+ B, 10 MILLIMOLES/L
0	6.35	6.35	0.2	0.2
6	6.35	6.35	1.2	0.6
17	6.20	6.30	4.5	2.4
24	5.75	5.72	9.0	6.5
42	5.75	5.72	9.0	9.0

fects of boron on the reaction were not due to a difference in pH.

With more active preparations of the starch phosphorylase enzyme, it was possible to observe a measurable reaction in much shorter time than indicated in figure 2 and table III. Table IV is a summary table of 18 experiments in which 0, 1, 10, and 50 millimoles/l of boron were used in the digests. In these experiments various preparations of enzyme and concentrations of substrate were used. Consequently, the results are given as a percentage of the phosphate liberated in the control digest. This table shows that with different preparations of the enzyme and over a range of substrate concentrations, the conversion of glucose-1-phosphate to starch is inhibited by the presence of borate ions.

To characterize the nature of the inhibition of boron on this reaction, a series of experiments were carried out in which the concentrations of glucose-1-phosphate and boron were varied. A reciprocal plot of the velocity of reaction and substrate concentration is given in figure 3. From the plot of the reactions at different boron concentrations, it appears that the inhibition is one in which the inhibitor (boron) complexes with the substrate (glucose-1-phosphate) and in so doing reduces the reactive substrate concentration, at the more dilute levels, to a value that is limiting the reaction. It can be seen that if higher concentrations of substrate were not used in this experiment, the results would indicate a competitive type inhibition. The Michaelis constant for the association of substrate and boron cannot be determined by enzymological measurement. If this constant were known for the glucose-1-phosphate and boron, it would be possible to substitute the value into the proper equation and the problem would be reduced to the Michaelis-Menten equation in its simplest form. The curves in figure 3 are similar to the example given by Friedenwald and Maengwyn-Davies (11) for coupling of inhibitor and substrate. Considering the ability of boron to complex poly-hydroxy compounds, this type of inhibition seems possible.

Since glucose-1-phosphate forms a weakly ionized compound with borate and glucose forms a strongly ionized compound (17, 18), it was thought that boron might also alter the phosphorylation of glucose by influencing the hexokinase reaction. The experiments

TABLE IV

SUMMARY OF 18 EXPERIMENTS SHOWING THE INFLUENCE OF BORON ON THE IN VITRO CONVERSION OF GLUCOSE-1-PHOSPHATE TO STARCH BY STARCH PHOSPHORYLASE ENZYME *

CONC, MILLIMOLES/L	MG P/DIGEST AS % OF CONTROL
0	100
1	94 ± 5.5
10	78 ± 5.5
50	50 ± 18

* The temperature and pH were constant in all experiments. Because of the difference in the activity of various enzyme preparations extracted in the course of this work the time of measuring the reaction varied between experiments from 30 min to 4 hrs.

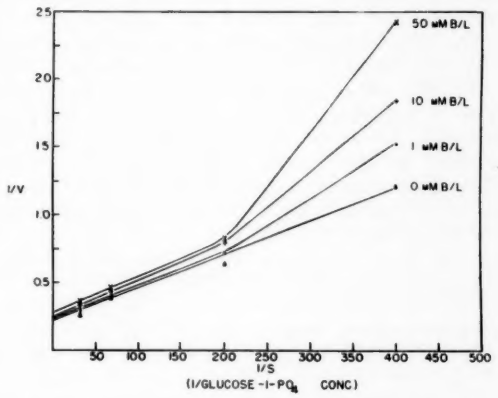

FIG. 3. The reciprocal plot of glucose-1-phosphate concentration vs rate of reaction.

conducted to test this hypothesis were carried out in vitro with hexokinase from yeast (Crude type II, Sigma Chemical Co.), and from pea seedlings and cotyledons. The method of separation of the insoluble or mitochondrial fraction from pea seedlings and the soluble fraction from pea cotyledons as well as the method of essay for hexokinase activity used in these experiments are described by Saltman (25). The reaction mixture of 1.5 ml contained 0.01 M adenosine triphosphate, 0.0033 M glucose, 0.01 M $MgCl_2$, 0.06 M TRIS (tris hydzoxymethyl-aminomethane) buffer, pH 8.0, enzyme and H_3BO_3 at 10, 50, and 100 millimoles/l. The control reaction did not contain boric acid. There was no effect of borate ion on the action of hexokinase in phosphorylating glucose. With yeast hexokinase 1.4 millimoles of glucose were phosphorylated per control reaction in 10 minutes at 30° C, whereas the reactions containing the boron, 1.5, 1.6, 1.4 millimoles of glucose were phosphorylated in the same time and at the same temperature. The reactions were duplicated and the experiments were repli-

cated twice. The standard errors of all the values were within ± 0.2 millimoles glucose. Glucose was estimated by a modification of the glucose oxidase methods[3] of Whistler et al (28), and Comer (2).

With the mitochondrial fraction from pea seedlings the glucose phosphorylated in 30 minutes at 35° C was 1.7 millimole per reaction for the control and 1.7, 1.7, 1.8 millimole per reaction for 10, 50 and 100 millimole/l of boron.

The soluble fraction of hexokinase from pea cotyledons was only one-third as active as the insoluble fraction from the seedlings; however, there was no difference in the amount of glucose phosphorylated between no boron and 10, 50 and 100 millimoles/l of boron.

Discussion

In the recent review of the physiological action of boron in higher plants (7), it was pointed out that 15 distinct postulated roles of boron have appeared in the literature. Many of these roles can be understood more fully when evaluated in terms of the concept that boron increases the translocation of sugars. The validity of this concept has been borne out by several kinds of experimental results (6, 26). The inability, however, to elucidate the mechanism of action has resulted in several working hypotheses (7). That these have remained hypotheses denotes the complexity of the problems associated with organic transport in plants. As is often the case, a different approach to the same problem is fruitful in understanding physiological problems such as this one.

A study of the effect of boron on the starch phosphorylase system seemed to be the best approach at this time. Winfield (29) had previously investigated the influence of boron on starch phosphorylase as well as potato aldehyde oxidase and tyrosinase. His report showed no effect from boron on the latter two enzymes in an in vivo study. In other experiments conducted with starch phosphorylase enzyme preparation, Winfield reported a 21 % inhibition of the synthesis of starch from glucose-1-phosphate by a 0.1 M concentration of boron. This is approximately 6,000 ppm, six times the highest concentration used in the experiments reported in this paper. With the same concentration of boron, Winfield showed a 17 % increase in the rate starch was degradated to glucose-1-phosphate under the optimum condition for the reaction. He proposed that boron entered into the "condensation reaction of sugar molecules in the same fashion as does phosphate ion," and that "it seems unlikely that boron influences the starch $\rightleftarrows$ glucose-1-phosphate equilibrium in normal plants where phosphate concentrations would be much greater than borate." The data presented in this paper indicate that the equilibrium is not changed but rather the rate of reaching equilibrium is changed. The competition between borate and phosphate as postulated by Winfield does not seem likely; rather, his results, as well as the results presented in this paper, are thought to be caused by the formation of a glucose-1-phosphate-borate complex.

The effects of arylboric acids on the growth of roots from wheat plants has recently been investigated by Torssell (27). He attributes the root growth-promoting property of these compounds to a characteristic of the dihydroxyboron group which in some way regulates the growth of the cells. He further states that this characteristic is not associated with auxin effects, but rather with the ability of arylboric acid to complex the carbohydrates in the polysaccharide chains of the cell wall and slow down the stabilization of the wall. "When the normal stabilization of the cell wall is prevented by arylboric acids, it will result in an increased cell elongation."

Further results by Torssell (27), cited as "in press," indicate that phenylboric acid in concentrations $\leqq 10^{-3}$ M did not influence starch phosphorylase, α amylase, nor invertase. Boric acid at 10^{-3} M was also found by the present investigators to be ineffective on starch phosphorylase.

Reed (24) showed that the catechol oxidase activity in the phloem cells of boron-deficient celery plants was higher than in normal celery plants. The cortical cells of boron-deficient plants also had a higher oxidase and phosphatase activity than did similar cells in plants supplied with boron. Abnormally high rates of oxygen consumption were noted by MacVicar and Burris (19) in whole tissue homogenates and chloroplast suspensions from boron-deficient plants. They associated this with an increase in polyphenoloxidase activity as a result of boron deficiency. Of the reactions studied by these investigators, the oxidation of dihydroxyphenyl-L-alanine showed significant inhibition with 0.001 M boron concentration. Higher concentrations of boric acid (0.01 M to 1 M) inhibited ascorbic acid oxidase from cabbage leaves. They justify the high levels of boron by saying that "within the cells a particular compound may be highly concentrated in local areas. With cell-free preparations it may be necessary to reproduce this high local concentration in the entire medium to achieve the activity apparent in the intact cell." This point is well taken and was also the reasoning behind using high levels of boron in the experiments reported here.

In a recent review (7), other investigations were cited with respect to experimental evidence showing inhibition or stimulation from added boron on tyrosinase, catalase, invertase, and diastase. Gilbert and Swallow (8) found that Q-enzyme was inhibited by sodium borate even in the presence of activating ions.

It is recognized that the inhibition of the starch phosphorylase reaction by boron may be only one of the many reactions so influenced. Any enzymatic reaction involving a substrate capable of complexing boron may be influenced by the element. On the other hand, boron could be a specific inhibitor irrespective

[3] The components of these methods are sold by Worthington Biochemical Corp. under the trade name of Glucostat.

of the degree to which it forms a complex with a substrate.

Compared to the glucose-borate complex the glucose-1-phosphate-borate complex is weakly ionized. According to Kyme and Cohn (17) and Goodman et al (9) glucose-1-phosphate is elutriated from an ion exchange column more rapidly with a weak borate solution than are the hexoses. Sucrose also forms a very weakly ionized complex with borate and may be rapidly elutriated from an ion exchange column. The authors point out that there is in general a direct relationship between the retention of sugar or sugar-phosphate on an ion exchange column and the increase in conductivity of the complex when sugar and borate are mixed (18), as well as a change in the optical rotation and pH (16) proportional to the degree of complexing. McPherson and Percival (18) found that the addition of borate to sucrose depressed the conductivity which was indicative of no complex formation. Isbell et al (16), on the other hand, found that the addition of borate to sucrose caused a change in the specific rotation as well as the pH, indicating the formation of a complex. The sucrose-borate complex formed is presumed to be of the 1,3 diol type. Glucose-1-phosphate, irrespective of the limitations set by MacPherson and Percival (18), also forms a complex with borate. Kyme and Cohn (17) found a slight increase in the volume necessary to elutriate glucose-1-phosphate from an ion exchange column as the borate concentration was increased from 10^{-5} to 10^{-2} M.

Such information with regards to the differences in the degree of borate complexing is of interest when the results with hexokinase and phosphorylase are compared. Glucose, which complexes strongly with boron, was phosphorylated by hexokinase at the same rate with and without boron. On the other hand, the conversion of glucose-1-phosphate to starch by starch phosphorylase was partially inhibited by 10 to 100 millimoles of borate per liter.

For purposes of developing a working hypothesis that included the findings reported here and the now well established effects of boron on translocation of sugars, the relationship between sucrose phosphorylase and substrate explained by Gottschalk and referred to by Hassid (15) is pertinent. For bacterial sucrose phosphorylase to synthesize or hydrolyze sucrose, a particular spatial arrangement of the substrate molecule is necessary. In order to satisfy the specificity of this enzyme, the glucose acceptor (fructose in the case of the disaccharide sucrose) must possess adjacent to the glucosidic oxygen an –OH group *cis* disposed and co-directional to the –OH group of the glucose part of the molecule. As pointed out by Zittle (30) borate ions also form compounds with cyclic diols that have *cis* hydroxy groups. In these experiments the added borate could combine with the substrate, glucose-1-phosphate, and effectively reduce the concentration of the substrate available for enzyme action to a lower, limiting value. This appears to be the case as shown by the double reciprocal plot in figure 3. The equilibrium value for the reaction between boron and a diol is dependent on the molar ratio of boron to diol as well as the hydrogen ion and water concentration (16). If non-borated substrate is removed from solution by enzymatic condensation onto existing starch molecules, the borated substrate $\rightleftarrows$ non-borated substrate equilibrium will shift to the right, and in time all the available substrate will enter into the enzymatic reaction. This could explain why the rate of reaching equilibrium and not the final equilibrium value in vitro is influenced by borate ions.

At any one time the quantity of soluble carbohydrates moving in the translocating tissue is a function, in part, of the quantity of soluble carbohydrate in the cells of the leaves. This would be true for carbohydrates synthesized in the leaf cells or added in some manner such as injection or infiltration. If there exists in the cells an inhibitor which prevents or slows down the condensation of soluble carbohydrates to starch, then it seems reasonable to assume that within a period of time, more of the carbohydrate will move out of the leaf cells to other plant parts than if the inhibitor were not present. This hypothesis could account for the translocation effect observed when boron is added to the sugar in "feeding" experiments (6, 22), or when boron is included in the mineral nutrient solution used for growing plants as compared with boron deficiency. Sisler et al (26) observed that the characteristic symptoms of brittleness in tomato appeared eight days after removing boron from the nutrient solution. After only four days, however, these plants showed a decrease in the amounts of C^{14} labelled carbohydrates that were translocated as compared to normal plants.

As a result of the present experiments and their interpretation, it is proposed that one of the ways in which boron may bring about an increase in translocation of sugars in plants is by decreasing the enzymatic conversion of glucose-1-phosphate to starch. With an increase in the steady state concentration of glucose-1-phosphate, the amount that may be available for other reactions, such as the synthesis of sucrose or other hexose phosphates, is increased. An increase in these soluble carbohydrates in situ may therefore result in an increase in translocation from the site of synthesis to some other plant part.

Summary

Bean leaves infiltrated with 4 % glucose after a dark period synthesized more starch than leaves infiltrated with 4 % glucose and various levels of boron. Analysis for boron concentration in the leaves indicated that the level of the element was within physiological range for plants.

In vitro studies in the glucose-1-phosphate $\rightleftarrows$ starch reaction show that boron influences the rate of the reaction but not the final equilibrium.

A plot of 1/rate of reaction vs 1/glucose-1-phosphate concentration indicates that, at low levels of substrate, the boron combines with the substrate and influences the rate of starch synthesis.

Because boron influences the synthesis of starch, a hypothesis is presented which could explain why translocation of sugars in plants is increased when the element is present in sugar "feeding" and in C^{14} carbohydrate synthesis experiments.

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BRIEF PAPERS

THE EFFECT OF GIBBERELLIC ACID ON ENZYME ACTIVITY AND OXYGEN UPTAKE IN BEAN PLANTS (PHASEOLUS VULGARIS)[1,2,3]

L. E. WELLER, S. H. WITTWER, M. J. BUKOVAC AND H. M. SELL

DEPARTMENTS OF AGRICULTURAL CHEMISTRY AND HORTICULTURE,
MICHIGAN STATE UNIVERSITY, EAST LANSING, MICHIGAN

Gibberellic acid, a tetracyclic dihydroxylactonic acid, $C_{19}H_{22}O_6$, produces marked shoot elongation in many plants (2, 3, 11, 12, 13). Unlike other auxins, its stimulation of growth of intact plants often results in substantial increases in height, and in fresh and dry weights (2). Brian (2) noted that the ash, nitrogen, phosphorus, potassium, total soluble carbohydrates, and carbon increased in the shoots and decreased in the roots of gibberellic acid treated wheat and pea plants. For the entire plant there was a net increase in these constituents. In rice seedlings Yabuta et al (13) found no difference in the ash, reducing sugar, total nitrogen, or total weight, but the treated plants contained less chlorophyll and total sugar. Wittwer et al (11) recently demonstrated that gibberellin will induce parthenocarpic fruit development in the tomato and earlier flowering in several crops. Kato (5) has reported significant increases in oxygen and water uptake of pea stem sections treated with gibberellin. Little information has been reported on the effect of gibberellic acid on plant metabolism; therefore, certain enzyme systems and rates of oxygen uptake of intact bean plants treated with gibberellic acid were studied.

Bean seeds var. Blue Lake (Stock no. 42335, Roger Bros. Seed Co., Inc., Idaho Falls, Idaho) were sown in quartz sand in a greenhouse. After 8 days the newly emerged seedlings were transferred to aerated solution cultures (standard Hoagland). Plants were grown during the long days (15- to 16-hour photoperiod) of early summer; temperature variations from night to day were 10 to 15° C. Ten microliters of an aqueous solution containing 10 micrograms of gibberellic acid was delivered from a pipette to the apex of the epicotyl at the time the primary leaves were fully expanded. Three replications of both treated and non-treated plants were utilized for all studies.

For enzyme determinations, the plants were harvested 96 hours after gibberellic acid was applied, separated into leaves, stems, and roots, frozen immediately, and maintained at −20° C until needed. Extracts (triplicate) of the leaves (10 plants), roots (10 plants), were prepared by macerating in a Waring Blendor for 5 minutes with 40 ml of water, and for the stems (20 plants) 20 ml of water was used. The solutions were permitted to stand for ½ hour. The solids were then removed by centrifugation and the supernatant liquid filtered. An aliquot of the filtrate was used for the enzyme determination.

The methods employed for the determination of the enzymes were those of Sandstedt et al (1, 7, 8) for α-amylase and β-amylase; Sumner et al (9) for phosphorylase; Ignatieff and Wastenays (4) for phosphatase; and Kertesz (6) for pectin methyl-esterase.

Oxygen uptake of stem segments from the same lot was determined immediately following harvest. The oxygen uptake of the freshly prepared stem sections was measured in a Warburg respirometer (10) which was shaken at 80 rev/min at 30° C. Each

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TABLE I

EFFECT OF GIBBERELLIC ACID ON CERTAIN ENZYMES IN VARIOUS TISSUES OF BEAN PLANTS * (FRESH WT BASIS)

ENZYMES	LEAVES		STEMS		ROOTS	
	TREATED	NOT TREATED	TREATED	NOT TREATED	TREATED	NOT TREATED
α-Amylase	Trace	Trace	Trace	Trace	Trace	Trace
β-Amylase **	81.3	89.4	43.5	54.0	98.7	72.1
Phosphorylase	Trace	Trace	Trace	Trace	Trace	Trace
Phosphatase †	4.5×10^3	3.36×10^3	5.13×10^2	4.69×10^2	Trace	Trace
Pectin methyl-esterase ††	0.52	0.48	0.16	0.22	26.4	59.4

* Values represent averages of 3 replications with the exception of pectin methyl-esterase which are the averages of 2 replications.

** Mg soluble starch converted to maltose by the enzyme per gm tissue in 1 hr at 25° C and pH 4.5.

† Mg phosphorus liberated from β-glycerophosphate by the enzyme per gm tissue in 1 hr at 25° C and pH 5.8.

†† Mg methoxyl group liberated from pectin by the enzyme per gm tissue in 1 hr at 25° C at pH 7.0.

flask contained approximately 200 mg of stems cut in 5-mm lengths and suspended in 2 ml of 0.1 M phospate buffer solution at pH 5.7. The experiments were run in an air phase 1 hour after equilibrium had been established.

At the time of harvest the overall shoot length of the treated plants was more than double that of the non-treated plants, while the roots of the former were about 2/3 the length of the roots of the control plants. Most of the observed elongation of the treated plants resulted chiefly from the extension of the internodal regions. No difference was observed in the total fresh and dry weights between the treated and non-treated plants.

The data from the enzyme determinations expressed on a fresh weight basis indicated the following trends: Only traces of α-amylase and phosphorylase activities (table I) were detected, and no differences could be ascribed to treatment with gibberellic acid. β-Amylase activity was higher in the extracts of leaves and stems of the non-treated plants; however, plants treated with gibberellic acid showed higher β-amylase activity in the roots. Significantly higher phosphatase activity was present in the extracts of leaves and stems of the treated plants. There was less pectin methyl-esterase activity in the roots of the treated plants with no difference in the stems and leaves.

On a plant part basis (table II) the uptake of oxygen by the stems (the internode between the primary leaves and the first trifoliate leaf) was increased sevenfold following treatment with gibberellic acid, while with the epicotyl segment between the primary leaves and the cotyledons, the microliters of oxygen absorption was doubled. Expressed on a fresh weight basis the oxygen uptake by the first internode of the treated plants was less than the non-treated plants, while no difference was observed with the internode between the cotyledons and the primary leaves. Kato (5), using pea stem sections suspended in gibberellic acid solutions (10 mg/liter) was unable to detect any difference (dry weight basis) in respiration in sections prepared from the first and second internode; however, oxygen uptake was increased in sections prepared from the third internode.

TABLE II

EFFECT OF GIBBERELLIC ACID ON OXYGEN UPTAKE BY THE INTERNODES OF BEAN PLANTS

PLANT TISSUE	O_2 UPTAKE, μL/HR AT 30° C			
	PLANT PART		TISSUE, 100 MG FRESH WT	
	TREATED	NOT TREATED	TREATED	NOT TREATED
First internode	121	18	40	71
Internode (epicotyl below primary leaves	44	22	12	14

SUMMARY

Young bean plants 96 hours after the application of 10 micrograms of gibberellic acid to the apex of the epicotyl showed on a fresh weight basis that phosphatase activity was increased in the leaves and stems of the treated plants. No differences could be detected for α-amylase and phosphorylase. Treatment with gibberellic acid resulted in a decrease in activity for β-amylase in the leaves and stems, for pectin methyl-esterase in the roots, for oxygen uptake in the first internode, and an increase for β-amylase in the roots. On a plant part basis, however, the oxygen absorption was greater in the internodes of the treated plants.

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A SPECTROPHOTOMETRIC ASSAY OF THE HILL REACTION WITH FERRICYANIDE[1,2,3]

DAVID W. KROGMANN[4] AND ANDRÉ T. JAGENDORF
McCOLLUM-PRATT INSTITUTE AND BIOLOGY DEPARTMENT, THE JOHNS HOPKINS UNIVERSITY,
BALTIMORE 18, MARYLAND

In studies of the photolysis of water by cell free preparations of photosynthetic tissue (Hill reaction), several assay systems have been used. The manometric method originally employed by Hill consists in measurement of oxygen evolution by a chloroplast preparation in the light, when an appropriate electron acceptor is provided. Ferricyanide and ferric oxalate or ferricyanide alone have frequently been employed as electron acceptors for the manometric measurement. Inherent difficulties in the manometric assay lie in the prolonged incubation of the chloroplast material with high concentrations of the electron acceptor and in the low sensitivity. In 1950, Milner et al (1) devised a rapid and extremely sensitive spectrophometric assay in which the decolorization of the dye 2,6-dichlorobenzenone indophenol, attendant to its reduction by illuminated chloroplasts, is measured. To eliminate some of the methodological differences encountered in comparing dye and ferricyanide reduction by the Hill reaction, a spectrophotometric assay for ferricyanide reduction has been devised. This spectrophotometric assay is felt to be superior to manometric measurements of ferricyanide reduction since the prolonged time of measurement is avoided, while the sensitivity of the dye reduction is retained.

An illuminated chloroplast suspension is allowed to reduce ferricyanide. After the illumination period, ferric chloride and orthophenanthroline are added. The ferric ion is reduced by the light generated ferrocyanide, and the free ferrous ion forms a colored chelate with orthophenanthroline.

To establish the sensitivity and precision of the method, a concentration curve was run using potassium ferrocyanide as the primary standard in the presence of excess ferric chloride. Sodium citrate was added to stabilize the ferric ion. The reaction mixture contained one micromole of ferric chloride, twenty micromoles of potassium citrate, and ten micromoles of orthophenanthroline. Aliquots of a 10^{-4} M potassium ferrocyanide solution were added to give a concentration range of 0.01 to 0.20 micromoles standard, and the reaction mixture was brought to a final volume of three ml. The diluent employed was the medium routinely used for Hill reaction assay, i.e., 0.05 M phosphate buffer pH 7, 0.4 M sucrose, 0.01 M KCl. The resulting color was measured at 510 millimicrons in the model B Beckman spectrophotometer. The ferrocyanide—through ferric chloride—gave a color response of 0.036 ± 0.003 optical density units per one hundredth micromole over the range indicated. A similar curve with ferrous sulfate as a standard, and thus avoiding the intermediate reduction of ferric iron, gave an identical response. That the standard ferrous sulfate solution was completely in the reduced form was demonstrated by the failure to increase the optical density of these samples by the addition of dithionite.

In testing this assay as a measurement of Hill activity, a spinach chloroplast preparation was employed. Spinach chloroplasts were prepared as previously described (2), and then sonicated in the Raytheon Sonic Oscillator until Hill activity to 2,3,6-trichlorophenol-indophenol was reduced to fifty percent of that of the original chloroplast preparation. The chloroplast sonicate was used to minimize differences

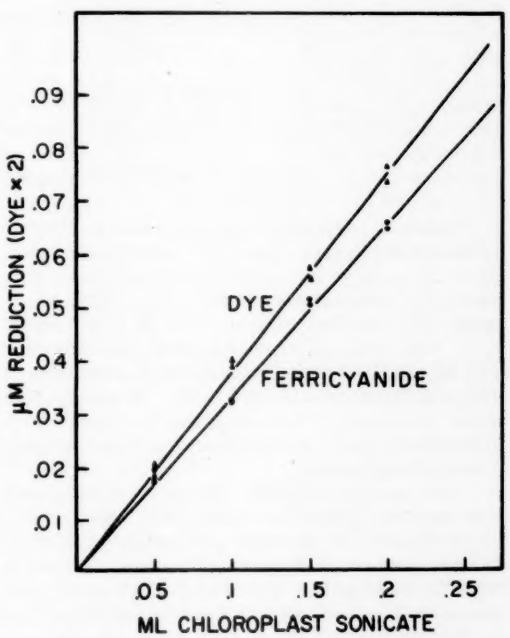

FIG. 1. Cuvettes were given a uniform sixty second exposure to saturating light. The chlorophyll concentration was 0.145 mg per ml of chloroplast sonicate. Micromoles of dye reduced are multiplied by two to make the dye reduction equivalent to that of ferricyanide, since dye reduction is a two electron change and ferricyanide a one electron change.

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[4] Predoctoral Fellow of the National Cancer Institute, Public Health Service.

in permeability of the chloroplasts to ferricyanide and dye. At final dilution before assay, the sonicated preparation contained 0.145 mg chlorophyll per ml. The measurement of the Hill reaction is accomplished as follows: chloroplast sonicate is mixed with phosphate sucrose KCl medium, and 0.1 ml of 0.01 M potassium ferricyanide is added. The optical density of the mixture is recorded. The test tube or cuvette is illuminated, then 0.1 ml of 0.2 *N* sodium citrate and 0.1 ml of 0.01 *N* ferric chloride in 0.1 *N* acetic acid are added, followed by 0.1 ml of 0.1 M orthophenanthroline in absolute alcohol. Allowing four minutes for complete color development, the optical density is compared to that of a control cuvette which had not been illuminated, but is otherwise identical.

In the figure, ferricyanide reduction is seen to be proportional to the concentration of chlorophyll added, and only 12 % less than the dye reduction achieved under similar conditions. Dye reduction experiments were carried out as described previously (2). Ferricyanide reduction is also found to be directly proportional to the duration of exposure to saturating light.

The method appears to be usable in measuring the reduction of ferricyanide by the Hill reaction, at low oxidant concentrations, over short periods of time. The substitution of quinones for ferricyanide proved impractical due to complicating nonenzymatic reactions between the quinones and ferric chloride.

Summary

A procedure is described for the spectrophotometric measurement of the reduction of ferricyanide by the Hill reaction. After illumination of the chloroplast preparation in the presence of ferricyanide, ferric chloride and orthophenanthroline are added. The resulting color is measured at 510 millimicrons. Ferricyanide reduction by sonicated chloroplasts is found to be proportional to chlorophyll concentration, length of light exposure, and is approximately equivalent to dye reduction.

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OXIDATIVE PHOSPHORYLATION BY SUBCELLULAR PARTICLES FROM CABBAGE[1,2,3]

HUGH T. FREEBAIRN[4] and LEMAR F. REMMERT

Department of Agricultural Chemistry, Oregon Agricultural Experiment Station

and

Department of Chemistry, Oregon State College, Corvallis, Oregon

Oxidative phosphorylation by plant particulate preparations has been shown by a number of workers (2, 4, 5) using a variety of preparatory procedures and incubation mixtures. Fixation of inorganic phosphate (Pi) occurred with any one of a number of citric acid cycle intermediates added as substrate. The highest P/O ratio, obtained with α-ketoglutaric acid as substrate, was above 3.0 (4). This report concerns a procedure for obtaining unusually large amounts of O_2 and Pi uptake with particulate preparations from cabbage.

Inner portions of young cabbage heads, harvested less than two weeks previously, were chopped into pieces smaller than one cubic centimeter. Each of six 110-gm portions of chopped cabbage was blended in three 5-second periods with 60 ml of a solution containing 1 M sucrose, 0.01 M Versene, and 0.1 M potassium phosphate buffer at pH 7.2. Blending was done in a Waring blendor as described previously (1). Each of the 6 homogenates was strained through 6 layers of 40-grade cheesecloth into the same container, and the pH of the solution was adjusted to 7.0 with potassium carbonate. The homogenate was centrifuged, first at 3,000 × g for 10 minutes, and then at 12,000 × g for 15 minutes. The fraction sedimented at 12,000 × g was washed into a single tube with 35 ml of a medium containing 0.6 M sucrose and 0.075 M potassium phosphate buffer at pH 6.9. This tube was centrifuged at 12,000 × g and the precipitate was transferred, with the aid of a small amount of fresh wash medium, to a mat of fine Pyrex glass wool in a Gooch crucible. While on the mat, globules of precipitate were dispersed into successive portions of wash medium by gently stirring with a glass rod. Each portion of medium was drawn through the mat by applying suction. Finally, the entire suspension was again filtered through the glass wool mat and diluted to the volume needed for distribution to the Warburg flasks. This use of a glass wool mat involved very little abrasive action but gave suspensions which permitted uniform aliquoting. The preparative procedure was conducted within the temperature range of 0 to 5° C.

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[3] Presented in partial fulfillment of the requirements for the Ph.D. degree at Oregon State College.

[4] Present address: Department of Plant Biochemistry, University of California, Riverside, California.

Results from a number of typical experiments are presented in table I. Observations made in these, and other experiments may be summarized as follows: 1) In general, fresh crisp cabbage broke up most rapidly and easily during the initial blending. Such cabbage usually gave preparations characterized by higher P/O ratios than those obtained when older, flaccid cabbage was employed. 2) Addition of hexokinase appeared to be generally unnecessary for preparations yielding high P/O ratios. Added hexokinase raised the P/O ratio in some experiments in which this ratio was otherwise low. In a similar manner, added cytochrome c appeared to stimulate O_2 and Pi uptake only in the case of preparations characterized by relatively low P/O ratios. These relationships probably depended upon the extent to which the integrity of the subcellular particles had been disturbed during blending of the cabbage and subsequent preparative steps. Millerd et al (5) also suggested that the cytochrome c requirement of subcellular particles is associated with the degree to which their integrity has been maintained. 3) Short incubation periods generally led to higher P/O ratios than did longer periods. Presumably the phosphorylation system suffered damage when incubated at 30° C in the abnormal environment provided by the medium. Despite this, incubation was often continued for periods longer than those required for measurable phosphorylation, in order to obtain more Pi uptake and greater accuracy. 4) A significant decrease in O_2 uptake was observed during the first 60 minutes of incubation upon lowering the initial Pi concentration in the flasks to less than 0.031 M. An initial concentration of 0.047 M was therefore used routinely to prevent the Pi level from limiting oxidative (and therefore phosphorylative) activity later in the experiments after some Pi esterification had occurred. 5) Highest substrate-induced O_2 and Pi uptakes were obtained with added DPN, TPN, ATP, glutathione, and Mg^{++} and Mn^{++} ions. 6) Malonate at 0.01 M caused, in a number of experiments, an average of 90 % inhibition of the O_2 uptake induced by succinate, and an average of 48 % inhibition of that induced by α-ketoglutarate. However, malonate did not significantly change the P/O ratio obtained with α-ketoglutarate as substrate, as might be expected if succinate oxidation with its low P/O ratio was inhibited. It is suggested that in the cabbage particles malonate exerted unknown effects, in addition to inhibition of succinic dehydrogenase. Malonate was not added routinely in the phosphorylation experiments. 7) No Pi was esterified in these preparations when incubation was conducted with nitrogen as the gas phase. 8) Phosphatase activity in these preparations was usually of little quantitative significance. For example, in the presence of 3.4×10^{-2} M ATP (110 micromoles/flask), an average of only 8 micromoles of Pi were produced in 70 minutes by the preparation. In such phosphatase studies glucose, hexokinase and fluoride were omitted and mannitol was added in replacement for sucrose (5).

TABLE I

OXIDATIVE PHOSPHORYLATION BY SUBCELLULAR PARTICLES FROM CABBAGE *

EXPT NO., INCUBATION TIME, SUBSTRATE AND AMT ADDED	NET O_2 UPTAKE	NET PI UPTAKE	P/O RATIO
	micromoles	*μgm atoms*	*micromoles*
1. 120 min α-Ketoglutarate, 20	50.5	96.5	1.9
2. 60 min α-Ketoglutarate, 10	12.3	34.0	2.8
3. 50 min α-Ketoglutarate, 10	13.7	33.0	2.4
4. 51 min α-Ketoglutarate, 10	9.0	27.0	3.0
5. 30 min α-Ketoglutarate, 20	6.4	24.4	3.8
6. 50 min α-Ketoglutarate, 10	11.2	42.5	3.8
Oxalacetate, 20	20.7	45.3	2.2
Fumarate, 20	18.7	42.8	2.3
Succinate, 10	10.2	23.6	2.3
7. 60 min Glutamate, 20	28.0	64.0	2.3

* Conditions of gas measurement were those described previously (1). The shaking rate was 120 oscillations per min. Corrections were made for O_2 uptake during the equilibration periods. The reaction medium in the Warburg flasks contained subcellular particles from cabbage (0.22 to 1.4 mg protein N per ml); 0.004 M $MgSO_4$; 0.0016 M $MnSO_4$; 25 ppm terramycin; 3.2×10^{-3} M K_2ATP (adenosine triphosphate); 0.375 M sucrose; 0.047 M potassium phosphate, pH 6.9; 2×10^{-4} M DPN (diphosphopyridine nucleotide); 2×10^{-5} M TPN (triphosphopyridine nucleotide); 2.5×10^{-3} M glutathione; 0.02 M NaF; 0.10 M glucose; and 0.075 mg cytochrome c and 0.156 mg yeast hexokinase (Pabst product, 28,000 K.M. units per gm at 30° C) per ml. Concentrations of substrates (potassium salts) are indicated in terms of micromoles per 3.2 ml; i.e. per flask. Inorganic phosphate (Pi) determinations were made by the "isobutyl alcohol colorimetric method" of Pons et al (6).

As shown in table I, P/O ratios between 2 and 3 were obtained with oxalacetate, fumarate, succinate or glutamate as substrate. P/O ratios above 2 with succinate probably depended upon concomitant oxidation of substrates derived from succinate, and upon the higher P/O ratios associated with the other oxidative steps. Krebs et al (3) initially obtained similar ratios upon adding succinate to liver, heart and kidney homogenates. P/O ratios above 3 were obtained only with α-ketoglutarate as substrate, as noted previously (3).

Between 20 and 100 micromoles of Pi were esterified in these experiments, amounts which are larger than those commonly reported for preparations from plants. Further work (unpublished results by L. F. Remmert and I. M. Pierovich) has demonstrated that esterification of 80 to 100 micromoles of Pi can be obtained in a high percentage of experiments with the cabbage preparations

Summary

Subcellular particles from cabbage were found to catalyze oxidative phosphorylation with P/O ratios as high as 3.8. Large amounts of inorganic phosphate were esterified in some of the experiments.

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American Society of Plant Physiologists, *Northeastern Section:* On May 3rd and 4th, 1957, the twenty-second Annual Meeting of the New England Section of the American Society of Plant Physiologists was held at the Brookhaven National Laboratories, Upton, Long Island, New York. Chairman Martin Gibbs presided.

The meetings were attended by approximately 140 registrants, the largest number to attend any New England Section meeting to date. Twenty-two papers were presented, most of which were concerned with metabolism. After the buffet dinner, Professor Ray F. Dawson, Columbia University, spoke about his experiences as a tobacconist.

Last year, at Brown University, it had been voted by the Section membership to extend a cordial invitation to the Society members of New Jersey and New York to join formally with the New England Section and elect representatives to the Executive Committee, with the understanding that the section would, upon their acceptance, be known as the Northeastern Section. By unanimous vote of the more than sixty members from New York and New Jersey present, the invitation was accepted.

Officers elected for 1958 were Dr. A. W. Galston, Chairman; Dr. T. T. Kozlowski, Vice-Chairman; Dr. H. J. Dyer, Secretary-Treasurer; Executive Committeemen: for New York, Dr. Ray F. Dawson and for New Jersey, Dr. Harold E. Clark.

Adjournment was followed Saturday afternoon by a tour of the laboratories of the Biology Department, the Van de Graaff generator, nuclear reactor, and other unique features, led by Drs. Anderson, Fuller and Shapiro of the Local Committee.

One clear measure of the success of any such meeting is the vigor of the discussions following the individual papers; this vigor is attested by the difficulty experienced in keeping to the allotted time schedule. Grateful appreciation is due the Administration of the Brookhaven National Laboratories, and in particular Dr. R. C. Fuller, chairman of the Local Committee, for their labors and hospitality.

Next year's meeting will be held at Yale University.—Hubert J. Dyer, Secretary-Treasurer.